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Molecular Toxicology of Chromatin: The Role of Poly(ADP-Ribose) In Gene Control.

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This research endeavor approaches "chromatin toxicity" in a selective manner. Environmental factors (both physical and chemical) exert a subtle long-term effect on cellular systems, that is distinguishable from the acute lethal effects of toxins or high doses of radiation. We focus our attention on the effect of "low-dose" toxicology which affects cellular behavior and alters cellular phenotype. Present concepts define the molecular basis of cellular phenotype as a carefully and specifically orchestrated composite of gene-expressions which contain both the determinants of cellular phenotype and life expectancies of specific cell types. Notably, chemical or radiation effects will inevitably result in cellulary phenotypic changes, the most pervasive being malignancy. The specific experimental approach commenced with the study of the nuclear polymer $(ADP\text{-ribose})_n$, which we predicted could lead to a more basic understanding of gene regulations (proto-oncogene and differentiation regulation being the defined field).

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Significant advances even in pragmatic aspects came from research that followed the outlined rationale. The specific experimental approach commenced with the study of the nuclear polymer $(ADP\text{-ribose})_n$, which we predicted could lead to a more basic understanding of gene regulations (proto-oncogene and differentiation regulation being the defined field).

Our previous work identified $(ADP\text{-ribose})_n$ as a unique nucleic acid-like molecule and demonstrated several modes of regulation of protein-DNA interactions within cellular systems (see preceding Progress Report). The antitransformation effect by small molecules emerged as a side product of our molecular biologically-oriented research, however, the importance of this by-product cannot be over-emphasized.

Many important questions remain unanswered, some are as follows:

1. How does the in vitro existing $(ADP\text{-ribose})_n$ content actually relate to biological regulations? This question now can be studied by newly developed methods.

2. What is the exact molecular mechanism of enzymatic synthesis of $(ADP\text{-ribose})_n$?

3. What is the molecular role of the polymerase-associated (coenzymic) DNA?

4. How do DNA-conformational changes (being the molecular basis of gene-expression regulation) correlate with the DNA-binding of poly(ADP-ribose) polymerase enzyme? Is the polymerase protein a specific DNA-sequence-searching protein or is it a part of a larger protein complex?

5. Finally, how do "antitransforming" non-toxic molecules influence cellular phenotype? Present hypothesis predicts that antitransforming molecules

may bind to specific DNA domains, which coincides (or are identical) with the DNA sequences required to serve as a "coenzymic" DNA for the polymerase. How does this mechanism operate by regulating "physiological" vs. transformation-proned DNA-topoisomeric structures?

Research carried out in 1985 addresses these problems in an inter-disciplinary manner, which is probably the only workable approach to these complex questions. It is of considerable significance that the laboratory at UCSF (Surge 101 area) has been reorganized in several basic aspects to meet the demands of technical requirements for experimentation related to questions 1 to 5 (above).

The following new facilities have been added:

1. Complete synthetic capability for the synthesis of any desired DNA-sequence.

2. Full capability to work with cells in culture, including full time personnel necessary for culturing. This side-steps the cumbersome collaborative arrangements of the past which previously slowed down progress;

3. Expansion of enzyme, peptides and DNA isolation methodologies (HPLC and immunochemical techniques).

4. Capacity to execute DNA-sequencing and various DNA-technologies (recombinant technology, cloning that is in a related but specific project aimed at the isolation of the polymerase gene) which are general techniques in this field (including large scale E. coli culturing).

The effectivity of these new techniques will become apparent within the ensuing periods of research.

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OF RESEARCH ACCOMPLISHMENTS FOR 1985

Simultaneous Determination of Mono and Poly(ADP-Ribose) In Vivo By Tritium Labelling And Direct HPLC Separation.

No 1.

Summary

A microanalytical method for the determination of cellular mono, oligo and poly(ADP-ribose) has been developed that does not involve enzymatic degradation of oligomers to ribosyladenosine. The method consists of separation of protein-bound mono, oligo and poly(ADP-ribose) adducts from soluble nucleotides followed by hydrolysis and quantitative isolation of AMP (derived from mono(ADP-ribose)-proteins), oligo and poly (ADP-ribose) by boronate affinity chromatography and subsequent isolation of these nucleotides by HPLC (18). Cis-diols in AMP, oligo and poly(ADP-ribose) are selectively oxidized by periodate, then reduced by [^{3}H]-borohydride. Conditions for the oxidation-reduction steps were optimized and tritiated AMP, oligo and poly(ADP-ribose) were quantitatively determined by radiochemical analysis of these components that were isolated by reversed-phase HPLC (18). One pmol ADP-ribose unit under standard conditions yields 2 to 2.2 x 10^3 cpm [^{3}H] and this sensitivity can be amplified by increasing the specific radioactivity of [^{3}H]-borohydride.

INTRODUCTION

Poly(ADP-ribose), which is formed from NAD and is present predominantly in cell nuclei, is a biological macromolecule of nucleic acid-like structure (1,2). Its cellular function is currently under extensive investigation (3-7). Determination of polymeric and monomeric ADP-ribose *in vivo* constitutes an important analytical biochemical problem in this area of research. Previously several analytical methods have been reported:

(a) isotope dilution by endogeneous poly(ADP-ribose) of labelled poly(ADP-ribose) or mono(ADP-ribose) (8,9).

(b) antigen-antibody reaction between poly(ADP-ribose) and anti-poly (ADP-ribose) antiserum (10,11,12) or conversion of mono(ADP-ribose) to AMP and estimation of AMP by a radioimmunoassay (13).

(c) fluorometric determination of ribosyladenosine derived from poly(ADP-ribose) (14,15,16).

(d) tritium labelling of ribosyladenosine derived from poly(ADP-ribose) (17).

We have recently developed novel high performance liquid chromatography (HPLC) methods for the simultaneous determination of *in vitro*-synthesized poly(ADP-ribose) and mono(ADP-ribose) on a reversed-phase column (18) and chain length analyses on an ion-exchange column (19). We now report a highly sensitive and specific method for the simultaneous analysis of polymeric and monomeric ADP-ribose *in vivo*, suitable for biochemical experimentation. This method differs from previous procedures (13,14,15,16,17) inasmuch as protein-bound mono and poly(ADP-ribose) are determined after microisolation of these compounds and, following hydrolysis to AMP and poly(ADP-ribose), analyses are performed

directly by borotritiation and HPLC (19) without chemical or enzymatic degradation of polymers into chemical subunits.

EXPERIMENTAL

MATERIALS

NAD, Tris-HCl, proteinase K, triethylamine, sodium periodate and potassium borohydride, were purchased from Sigma Chemical Co. (St. Louis, Mo., USA); [^{3}H]-$NaBH_4$ (Lot No. 2558124, 1.68 Ci/mmol) from ICN Radiochemicals (Irvine, Ca., USA); [^{14}C]-NAD (283 mCi/mmol) from Amersham (Arlington, Ill., USA); Boronic acid gel (Affi-gel 601) from Bio-Rad (Richmond, Ca., USA); Ammonium carbonate and glacial acetic acid (HPLC grade) from Baker (Phillipsburg, N.J., USA); and potassium phosphate (HPLC grade) from Fisher (Santa Clara, Ca., USA); methanol and acetonitrile (HPLC grade) obtained from Alltech (Deerfield, Ill., USA). All other chemicals used were reagent grade. Scintillation fluid (Aquasol) was obtained from New England Nuclear (Boston, Ma., USA), and cell culture supplies from the Cell Culture Facility of UCSF, San Francisco, Ca., USA. The 14C rat fibroblasts containing MMTV promoter, EJ-ras oncogen and a 4.8 kb Sma-Bgl II DNA sequence construct, isolated by Dr. William M. Lee (Dept. Microbiology and Immunology) were a gift from Dr. Alex Tseng (Cancer Research Institute).

HPLC

HPLC was performed with the following instrumental components: Waters Associates (Milford, Ma., USA) Model 6001 Solvent Delivery Pumps, Waters Model 680 Gradient Controller, Waters Model 730 Data Module, and Hewlett-Packard (Santa Clara, Ca., USA) Model 1040A High Speed Spectrophometric Detector. Chromatographic data were stored in a Hewlett-Packard Model 9121D Disc Memory System and plotted by a Hewlett-Packard HP 747A Graphic Plotter. HPLC eluate fractions were collected with an Isco (Lincoln, Ne., USA) Model

(Retriever No. III) Fraction Collector and counted on a Beckman (Palo Alto, Ca., USA) LS 3800 Scintillation Counter. The column employed was a Beckman-Altex (Berkeley, Ca., USA) analytical reversed-phase column (Ultrasphere ODS, 5 μm, 25 cm x 4.6 mm ID), with a precolumn packed with the same sorbent as the analytical column. Chromatography was carried out at ambient temperature. The buffer system employed was: Buffer A, 0.10 M potassium phosphate (pH 4.25); Buffer B, same as A but containing 20% methanol; Buffer C, 0.10 M potassium phosphate (pH 7.0), 1.0 M urea and 50% acetonitrile. The flow rate was 1.5 ml/min. throughout. Upon sample injection the gradient started from 100% A to 100% B in 20 minutes, using concave gradient curve 9. Elution continued at 100% B for an additional 2 minutes and then a linear gradient was commenced from 100% B to 100% C during 6 minutes, and elution was continued at 100% C for ca. 10 minutes.

Isolation of poly and mono(ADP-ribose) present in vivo from cells. Cultures of 14C rat fibroblasts were maintained in Dulbecco's MEM + 10% FCS + penicillin/streptomycin at 33°C in 5% CO_2 + air atmosphere. Doubling time of the cells was 24 hours and at the time of confluency cells were removed by trypsin treatment, sedimented at 1500 rpm (room temperature), washed twice by centrifugation in PBS and counted in a hemocytometer. Figure 1 shows the overall steps on the isolation of poly(ADP-ribose) from 14C rat fibroblasts. For a detailed experimental description, see Reference 19 . Briefly, the cell pellet was extracted with 20% (w/v) trichloroacetic acid to remove soluble nucleotides. For each extraction, the pellet was dispersed by sonication (power setting 2, four sets of 20 pulses on a Bronson Sonifier) with cooling in an ice-water bath followed by centrifugation at 4°C (3000 x g for 20 minutes). Extraction was repeated six times to assure complete removal of nucleotides which otherwise may be trapped in the acid-insoluble material. The protein-bound polymers

and monomers were isolated by successive base hydrolysis, proteolysis by proteinase K, followed by phenol extraction and phenyl boronate chromatography. Elution of poly(ADP-ribose) and of AMP (which derives from mono(ADP-ribose)) from the boronate column (7 cm x 0.7 cm) was done with 1 M triethylammonium acetate* (pH 6.3) instead of Tris-HCl. After freeze-drying the material was dissolved in distilled water and subjected to periodate oxidation and [^{3}H] sodium borohydride reduction (see below).

In vitro preparation of poly(ADP-ribose) standard. The method employed is described previously (18) except that for elution of poly (ADP-ribose) from the boronate column 1 M triethylammonium acetate (pH 6.3) was used instead of Tris-HCl.

Preparation of 2'-(5'-phosphoribosyl) adenosine 5'-monophosphate (PR-AMP) standard was done as described previously (18).

Tritium "labelling" of poly(ADP-ribose) and AMP.

(a) Oxidation of poly(ADP-ribose) and AMP with sodium periodate. Samples containing up to 50 nmol of cis-diol groups in 0.100 ml of distilled water were combined with 0.010 ml of 10 mM sodium periodate, vortexed and allowed to react in the dark at room temperature for 30 minutes. This reaction time assures complete oxidation of the 2',3'-cis-diols of the ribose moieties of the polynucleotide to the corresponding dialdehydes without breakage of the polymer backbone (18). The oxidized sample was immediately reduced in situ (see below).

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*In contrast to Tris-HCl, triethylammonium acetate quantitatively elutes poly and mono(ADP-ribose) from the boronate gel and, due to its volatility, can be removed by freeze-drying, thus eliminating the need for desalting.

(b) Reduction of oxidized poly(ADP-ribose) and AMP with [^{3}H]-sodium borohydride. The periodate-oxidized sample of the polynucleotide or AMP was chilled, buffered by addition of 0.005 ml of 0.10 M potassium phosphate (pH 6.6) followed by 0.005 ml of a 0.10 M solution of sodium [^{3}H]-borohydride in 0.10 M potassium hydroxide. The resultant solution, which was mildly basic (pH 8-9), was allowed to react in the dark at room temperature for 30 minutes. Excess reducing agent was then decomposed by addition of 0.025 ml of aqueous acetic acid (2.0 M) and the sample dried in the fume hood by a stream of nitrogen. In order to assure complete removal of exchangeable tritium, the dried sample was redissolved in 0.100 ml of aqueous acetic acid (0.50 M) and then redried, and this step was repeated once more. The dried sample was then dissolved in distilled water and an aliquot corresponding to 2 to 10 x 10^6 cells was injected into the HPLC system.

RESULTS AND DISCUSSION

The chemical reactions on which this method is based are: (a) the oxidation by sodium periodate of the 2',3'-cis-diol groups of the ribose moieties in poly(ADP-ribose)[†] and related nucleotides, wherein the ribose rings are opened by a splitting of the carbon-carbon bond of the cis-diol and two aldehyde groups are formed, and (b) the reduction by [^{3}H]-borohydride of these aldehydes to primary alcohol groups. In the reduction of each aldehyde group, a C-H [^{3}H] bond is formed which, being nonexchangeable with water hydrogens, constitutes a stable isotopic labelling.

The procedure for periodate oxidation - [^{3}H]-borohydride reduction which we employ represents a modification of a method previously reported

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[†] In poly(ADP-ribose) each monomeric unit contains two ribose moieties, but only one of these has a cis-diol available for oxidation.

for oxidation-reduction of monomeric ribonucleosides (21) and ribosyladenosine (17,22). Because our approach, unlike that of previous workers, is to oxidize poly(ADP-ribose) in its polymeric form without degradation, it was desirable to determine conditions for the reactivity of the polymer with sodium periodate. In a previous study (18) we showed that poly(ADP-ribose) indeed undergoes periodate oxidation - [^{3}H]-borohydride reduction without disruption of the polymeric backbone, but we did not determine temporal requirements for optimal conditions. As seen in Table I at a concentration of 0.5 mM (ADP-ribose) units and 1.0 mM sodium periodate the initial half-life of oxidation of the polymer (measured by sodium periodate uptake) is four times longer than that of adenosine and ten times that of AMP. However, the rate of reaction is still sufficiently fast that, at room temperature with 2 equivalents of periodate present, oxidation is complete within 30 minutes.

The time requirement for reduction by borohydride of the oxidized groups (dialdehydes) was determined by HPLC analyses of dialdehyde reduction. The reversed-phase HPLC system (18) readily separates poly(ADP-ribose) from nucleotides (Figure 2). Figure 2 also shows the elution peak identifying AMP which has been periodate oxidized and borohydride-reduced, denoted AMP*$^{\Delta}$, which we employ as a primary standard. Figure 3 presents our kinetic study of the reduction of periodate-oxidized AMP (AMP-dialdehyde) by borohydride to AMP* . At an initial concentration of 0.48 mM AMP-dialdehyde and 4.8 mM borohydride, at room temperature, pH 9, reduction to AMP* is rapid and complete within 10 minutes. As seen in Figure 3 at zero reaction time AMP-dialdehyde displays itself as a broad elution peak centered at ca. 4 min.

$^{\Delta}$nucleotides with * denotes [^{3}H]-labelling.

At a reaction time of 0.25 min. this broad peak is largely transformed to two new peaks. The first at retention time 4.90 min., the second at 7.45 min. The first of these new peaks is presumably the partially reduced species (i.e., with one aldehyde group still unreacted) while the second is the fully reduced product, AMP*. This becomes evident as the reaction progresses and at reaction time of 10 min. essentially all of the material is consolidated into the AMP* peak.

It is further worth noting that (a) a minor peak is observed at retention time 23.5 min. (Figure 3). We have observed that if the reaction mixture is unbuffered, this peak, which possibly represents a dimeric aldehyde species, increases in size although still remaining a minor component. Therefore, in order to minimize this minor product we include potassium phosphate (pH 6.6) in an amount equivalent to the potassium hydroxide in the carrier solvent of the borohydride reagent. Thus, when borohydride is added to the periodate-oxidized nucleotide, the resultant solutions has a pH of about 9, which is sufficiently basic to prevent decomposition of borohydride by aqueous protons, but not so strongly basic to catalyze aldehydic adducts. (b) Excess sodium periodate, which carries over from the oxidation step, does not interfere with the reduction reaction. Even when the amount of excess periodate was intentionally tripled, no inhibition of the borohydride reduction of the dialdehyde was observed. (c) If the borohydride reaction mixture is allowed to stand at room temperature for periods up to an hour, no decomposition of AMP* is detected in the HPLC.

Based on the rapidity of the borohydride reduction of AMP-dialdehyde, we estimate that a 30-minute reaction time would be ample for complete

reduction of periodate-oxidized poly(ADP-ribose) to poly(ADP-ribose)*. This was confirmed by reacting oxidized poly(ADP-ribose) with [^{3}H]-borohydride (in quantities analogous to those detailed above for AMP) for 30 min., isolating the polymer by HPLC (see below), determining its specific radioactivity, and then re-subjecting the material to a new cycle of reduction with a large excess of [^{3}H]-borohydride (100 x). No further incorporation of radioactivity (no change in specific radioactivity) was detected, proving that during the initial 30-minutes complete reduction was effected. In addition, the specific radioactivity of the poly(ADP-ribose)* was consistently the same as that of AMP*.

Concerning the HPLC behavior of the tritiated derivatives of the structural constituents of poly(ADP-ribose) (i.e., AMP*, PR-AMP*, and Adenosine*) on the ODS reversed-phase column, it is predictable that these molecules have shorter retention times than their unreacted parent compounds. Table II gives the retention times for adenosine, AMP and PR-AMP and their tritiated derivatives. The shorter retention times are expected because oxidation-reduction of the secondary hydroxyl groups of the ribose ring (2',3'-cis-diol) results in breakage of the ring into open chain primary alcohols. However, as we have previously shown (18), oxidation-reduction of poly(ADP-ribose) does not alter significantly its retention time in this elution system, contrary to the behavior of monomers.

For quantitation of poly(ADP-ribose) by conversion to poly(ADP-ribose)*, we simultaneously carry out generation of AMP* from AMP as primary external standard. Figure 4A shows the elution profile of 5 nmol AMP* as followed by radioactivity counts of the HPLC eluate fractions.

Repeated preparations of AMP* routinely gave us a total of about 2.1×10^6 cpm/nmol AMP*. At this specific radioactivity it is possible to detect subpicomol amounts of tritiated product. If additional sensitivity is required, [^{3}H]-sodium borohydride of higher specific radioactivity can be employed.

When analyzing poly(ADP-ribose) content of biological samples, we routinely ran parallel samples of AMP, subjecting them to the same reactions and monitoring the specific radioactivity of AMP* as an indication of complete labelling efficiency.

Figure 4B shows the elution profile of radioactivity of a double-labelled sample of poly(ADP-ribose)* (4 nmol) derived from [^{14}C]-poly (ADP-ribose) (generated by in vitro incubation of [^{14}C]-NAD with rat liver nuclei (18)). From this HPLC chromatogram, it is clear that [^{3}H]-labelling has occurred only where [^{14}C]-label is present, except for the first elution peak at about 2.5 min. (denoted as peak 1) which has virtually no retention time in the column. Even in blank samples containing no nucleotide such a tritium-containing peak is observed.

We have found that the elution profile and specific radioactivity of poly(ADP-ribose)* is not affected when the amount of poly(ADP-ribose) is decreased significantly relative to the excess sodium periodate and [^{3}H]-borohydride reagents. Thus, when we subjected 50 pmol of poly(ADP-ribose) to the same exact conditions as 5 nmol, the poly(ADP-ribose)* gave the same elution profile and specific radioactivity. However, peak 1, which does not derive from the polymer, is observed to be larger and constitutes the most intense peak in the chromatogram (not shown).

Since in biological samples the amount of poly(ADP-ribose) is not known prior to analysis, it is important that the amounts of excess reagents do not interefere with complete conversion to poly(ADP-ribose)*.

In Figure 4B, the double-labelled [^{14}C, ^{3}H] peaks are identified as follows: Peaks 2 and 4 are PR-AMP* and very short oligomers respectively, while peaks 3 and 5 are AMP* and poly(ADP-riobse)* respectively.

Figure 5 represents a typical chromatogram obtained from a biological sample containing poly(ADP-ribose) from 14C rat fibroblasts. Poly(ADP-ribose) from 1.08 x 10^8 cells was isolated and dissolved in 300 µl of water. A 100 µl portion was subjected to the periodate oxidation - [^{3}H] borohydride reduction procedure (see Experimental section). An aliquot of the [^{3}H]-labelled product corresponding to 4.5 x 10^6 cells was chromatographed. The [^{3}H] counts under the poly(ADP-ribose)* peak totalled 1.8 x 10^6 cpm, corresponding to 175 pmol poly(ADP-ribose) per 10^6 cells. The AMP measured as AMP*, which represents the mono(ADP-ribose) content of the cells, was 14.3 pmol per 10^6 cells. This means that one pmol ADP-ribose unit is equivalent with 2.2 x 10^3 cpm [^{3}H]. Since the method is very sensitive and reproducible, a much smaller number of cells than specified above is sufficient to yield quantitated amounts of AMP* and poly(ADP-ribose)* radioactivity.

The intracellular poly(ADP-ribose) concentrations reported by others (14,23-25) and obtained by different techniques in our laboratory (cf. 26) are in the same order of magnitude as results obtained by the present technique. However, comparison with the reported method based on ethenoadenine fluorescence (16) is difficult for several reasons. Cellular concentrations of ribosyl enosine are shown as relative fluorescence values and the range of detection (1-5 pmols) was calculated from the fluorescence of standards without analytical data from biological material (16).

Furthermore, the internal standard, as stated (cf. 16) was oligo-ADP-ribose, n_{aver} = 2.5, which has been eluted by HCl from the affinity column. As we have shown (19) the chromatographic behavior of oligo and poly(ADP-ribose) are significantly different and we also find (unpublished results) that polymeric ADP-ribose, with n_{aver} = 30 to 50 does not elute from boronate affinity columns by acidic eluants. Therefore, the possibility cannot be excluded that by acid elution of oligo(ADP-ribose) that has been isolated from biological material, some or all of long chain polymers remain on the affinity column and may escape detection.

These discrepancies, besides being attributable to the technical problem of failure to elute long polymers from affinity columns by acidic eluants (16), could reflect also cell-specific variations (27). This problem concerned with comparison of varying cell types is the subject of a separate report.

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FIGURE LEGENDS

Figure 2. Reversed-phase HPLC analysis of poly(ADP-ribose) and monomeric nucleotides. Fifty-µl aliquots were injected, containing standards. Chromatography was performed at room temperature and monitored by UV at 260 nm; with a recorder scale of 0.15 a.u.f.s. Arrows indicate the positions of compounds not included in the standard mixture but their elution times determined separately.

Figure 3. Reversed-phase HPLC monitoring of the borohydride reduction of periodate-oxidized AMP. Five hundred nmol of oxidized AMP was mixed with 5000 nmol of borohydride in 4.5 mM potassium phosphate, pH 9, in a final volume of 1100 µl. Aliquots (110 µl) were removed at specific times, immediately quenched by addition of 50 µl of 2.0 M acetic acid, dried, dissolved in 100 µl of water and a 30 µl portion (corresponding to 15 nmol of oxidized AMP) injected into the HPLC. Chromatography performed at room temperature with UV detector at 260 nm. The ordinate was 0.300 a.u.f.s. in each chromatogram. The reaction times are indicated on each plot.

Figure 4. Reversed-phase HPLC analysis of (A) AMP* and (B) poly (ADP-ribose)* derived from in vitro-generated poly(ADP-ribose). In (A) 5 nmol of AMP* in 20 µl of water were injected and fractions of the eluate were collected and counted every 0.5 min. In (B), a 20 µl sample containing

Legends to Figures (Continued)

4 nmol of poly(ADP-ribose)*, were injected and 0.5 ml/min. fractions of the eluate were collected and counted. The left ordinate represents [^{3}H] and the right ordinate [^{14}C].

1 = background peak

2 = PR-AMP*

3 = AMP*

4 = short chain $(ADP\text{-}ribose)^{*}_{n}$ = 4-12

5 = $poly(ADP\text{-}ribose)^{*}_{n}$ = 20-50

Figure 5. Reversed-phase HPLC quantitation of poly(ADP-ribose) present in 14C cells.

TABLE I

Initial half-lives of the Reactions of Sodium Periodate (1.0 mM) with 0.50 mM adenosine, 5'-AMP and poly(ADP-ribose) in distilled water at 25°C.[a]

Nucleotide	$t_{\frac{1}{2}}$ (min.)
Adenosine	1.0
5'-AMP	0.4
Poly(ADP-ribose)	4.0

[a] Progress of the reactions followed by decrease in UV absorbance at 310 nm as described (20).

TABLE II

Comparison of Retention Times of Parents and Tritiated Derivatives[a] of Structural Constituents of Poly(ADP-ribose).

Compound	Retention Time (min.)	
	Parent	Tritiated Derivative
PR-AMP	10.40	5.12
AMP	11.62	7.45
Adenosine	22.84	14.8

[a]periodate oxidized - [^{3}H]-borohydride reduced.

FIGURE 1

FLOW-SHEET FOR THE EXTRACTION AND DETERMINATION OF MONO AND POLY(ADP-RIBOSE)

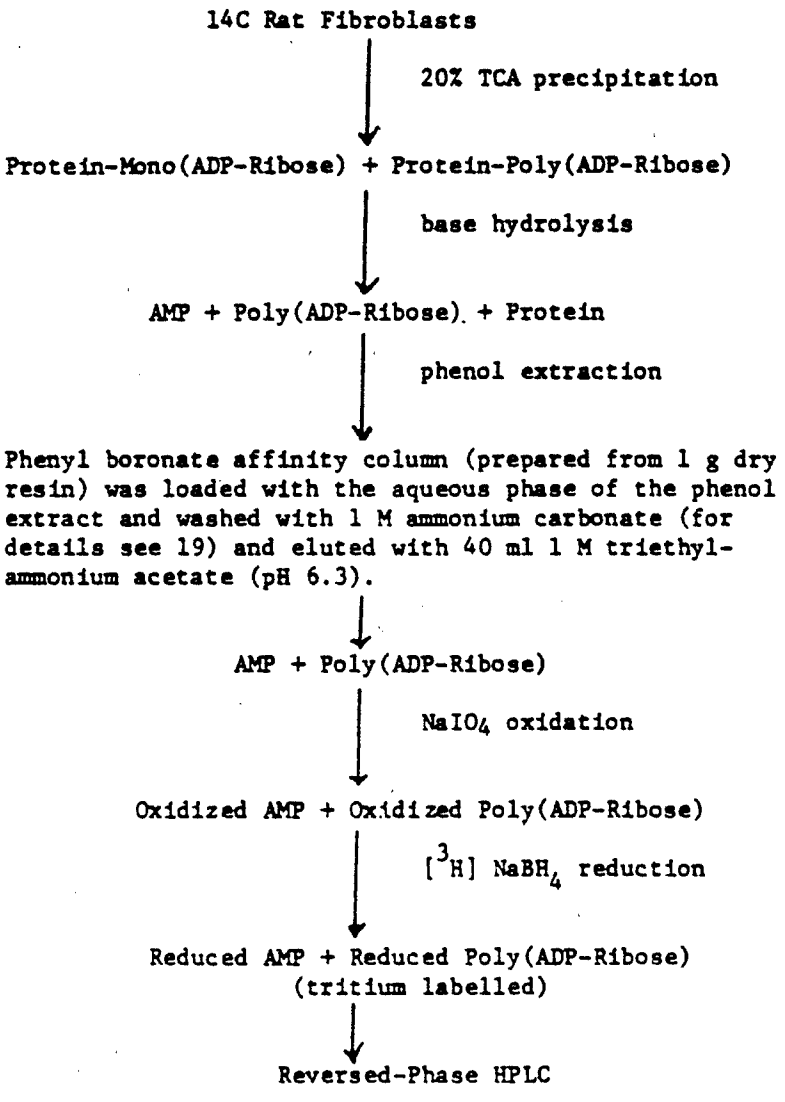


Fig 2.

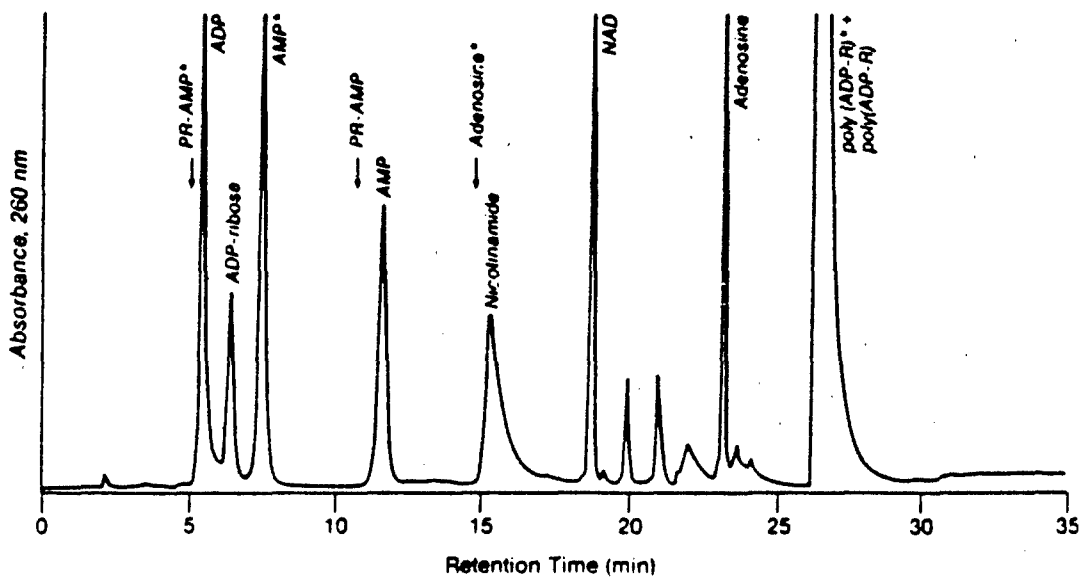

Absorbance, 260 nm
PR-AMP*
ADP
ADP-ribose
AMP*
PR-AMP
AMP
Adenosine*
Nicotinamide
NAD
Adenosine
poly (ADP-R)* + poly(ADP-R)
0
5
10
15
20
25
30
35
Retention Time (min)

Fig 3.

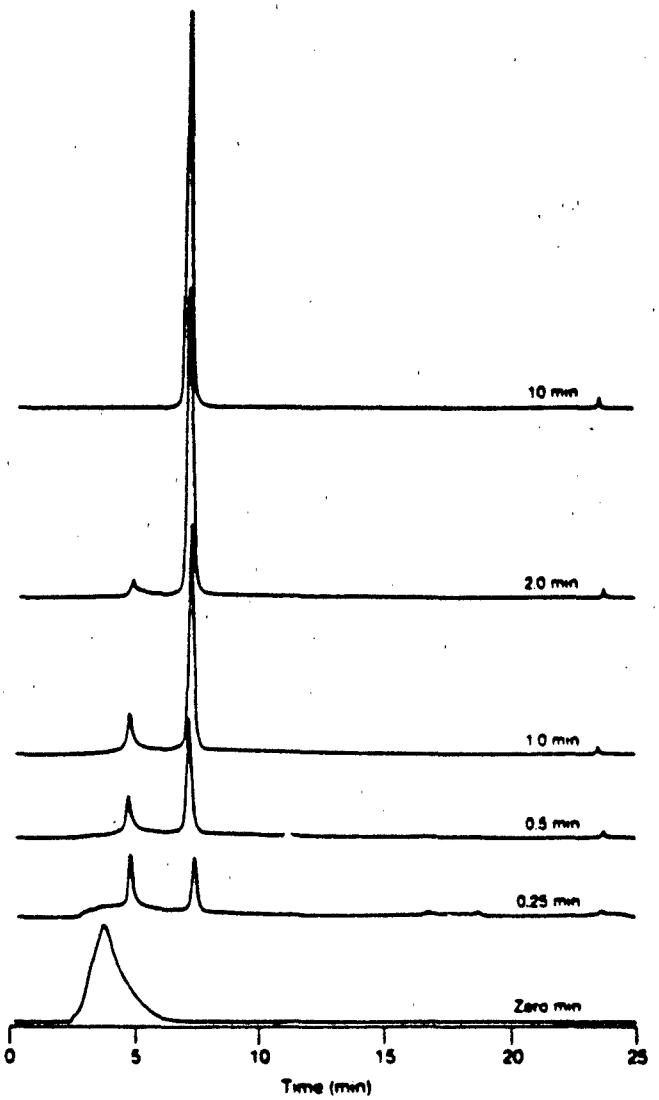

10 min
2.0 min
1.0 min
0.5 min
0.25 min
Zero min
0
5
10
15
20
25
Time (min)

Fig 4.

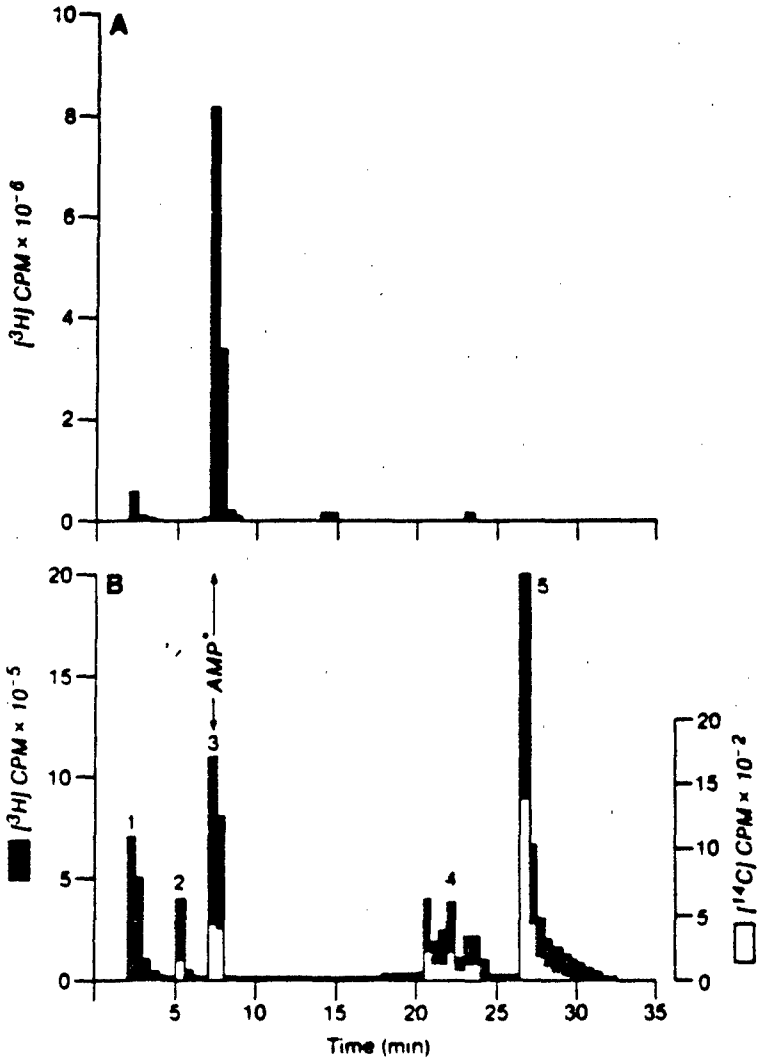
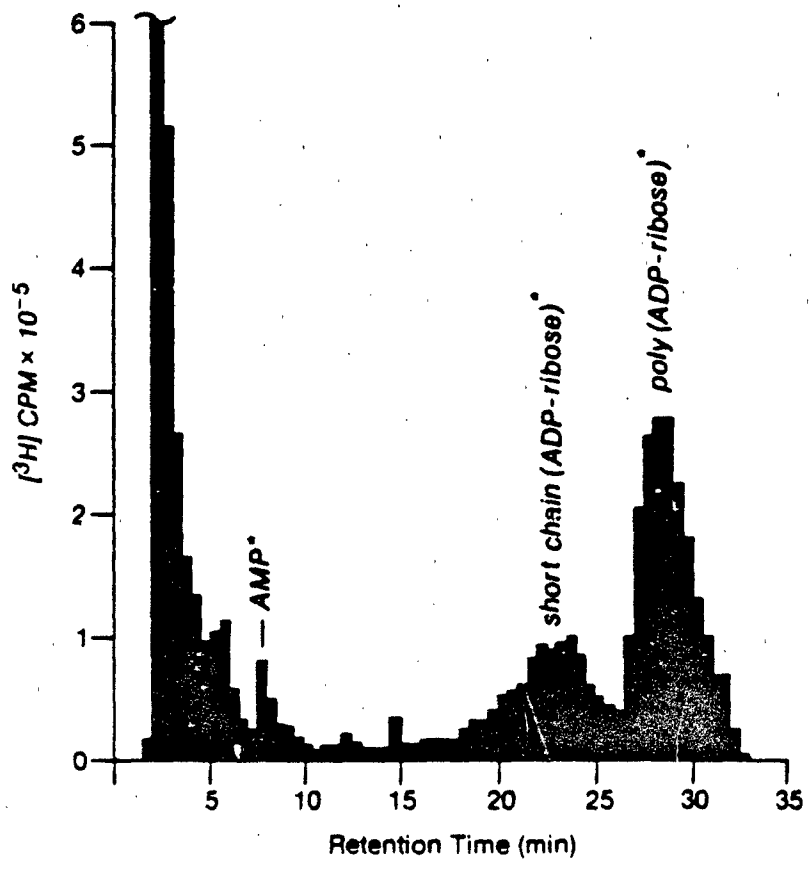


Fig 5.


[3H] CPM x 10-5
6
5
4
3
2
1
0
AMP*
short chain (ADP-ribose)*
poly (ADP-ribose)*
5
10
15
20
25
30
35
Retention Time (min)

Mechanisms of poly(ADP-ribose) polymerase catalysis; mono-ADP-ribosylation of poly(ADP-ribose) polymerase at nM concentration of NAD.

No 2.

Summary

Calf thymus and rat liver poly(ADP-ribose) polymerase enzymes, and the polymerase present in extracts of rat liver nuclei synthesize unstable mono-ADP-ribose protein adducts at 100 nM or lower NAD concentrations. The isolated enzyme-mono-ADP-ribose adduct hydrolyses to ADP-ribose and enzyme protein at pH values slightly above 7.0 indicating a continuous release of ADP-ribose from NAD through this enzyme-bound intermediate under physiological conditions. NH_2OH at pH 7.0 hydrolyses the mono-ADP-ribose enzyme adduct. Desamino NAD and some other homologes at nM concentrations act as "forward" activators of the initiating mono-ADP-ribosylation reaction. These NAD analogs at μM concentrations do not affect polymer formation that takes place at μM NAD concentrations. Benzamides at nM concentrations also activate mono-ADP-ribosylation of the enzyme, but at higher concentrations inhibit elongation at μM NAD as substrate. In nuclei, the enzyme molecule extensively auto-ADP-ribosylates itself, whereas histones are trans-ADP-ribosylated to a much lower extent. The unstable mono-ADP-ribose enzyme adduct represents an initiator intermediate in poly ADP-ribosylation.

It is generally assumed (cf. 1-4) that the chromatin-bound enzyme poly(ADP-ribose) transferase or polymerase (E.C. 2.4.99) transfers ADP-ribose residues of NAD to acceptor proteins, and notably in the isolated form the enzyme protein itself can serve as an acceptor in the presence of coenzymic DNA (15,26), resulting in auto-poly-ADP-ribosylation. Since proteins other than the polymerase protein also contain labelled ADP-ribose (1,2) following incubation of nuclei with labelled NAD it must be assumed that a transfer mechanism is operative from NAD either directly ADP-ribosylating "acceptor" proteins (e.g., histones), a reaction presumably catalyzed by the polymerase, or alternatively the auto-ADP-ribosylated enzyme could itself trans-ADP-ribosylate certain acceptor proteins. This question appears to be incompletely resolved. It is known from extensive studies from Hayaishi's laboratory (2,14) that once a polymer is formed on the enzyme it cannot be transferred to histones. Therefore, if a transfer occurs at all, it must happen at the mono-ADP-ribose level. It has also been demonstrated that histone-bound ADP-ribose can be elongated by purified poly (ADP-ribose) polymerase (5). It seemed almost immaterial what reaction led to a histone-mono-ADP-ribose adduct, since even Schiff base adducts of histones (6) served as elongation templates (cf. 5). It is now known that several, sometimes non-nuclear located, ADP-ribose arginyl transferases, distinct from poly(ADP-ribose) polymerase, can transfer ADP-ribose to histones or casein and probably to other proteins with apparently no rigorous acceptor specificity (cf. 3) and the histone adducts thus formed can be further elongated by poly(ADP-ribose) polymerase (7). Mechanistic interpretation of the enzymology of poly ADP-ribosylation and of important supra-molecular events in chromatin that appear to coincide with shifts in

protein patterns of ADP-ribosylation (8,9) depend on a more detailed understanding of the initial steps of ADP-ribosylation at the poly(ADP-ribose) polymerase level (10). Pursuing this question, the existence of an early product (11) was indicated. This problem was further studied by incubation of the purified enzyme with 1 μM NAD for 20 seconds (at 37°C) followed by isolation of mono and oligo ADP-ribose that was bound to the enzyme protein, thus leading to the conclusion that the enzyme contained multiple "initiator" sites (12). However, a possibly unusual nature of the initiator site has not been considered. We have employed nM concentrations of NAD as substrate for the poly(ADP-ribose) polymerase of various nuclei and found that almost exclusively mono-ADP-ribose adducts were synthesized (13) indicating that below μM concentration of NAD the polymerase appeared to catalyze mono-ADP-ribose transfer. Following this experimental approach we report here that purified poly(ADP-ribose) polymerase from two sources catalyzes the auto-mono-ADP-ribosylation of the enzyme protein resulting in highly unstable monomer adducts that especially under alkaline conditions, reported earlier to be optimal for the polymerase enzyme (cf. 1,2), release ADP-ribose from NAD apparently accounting for the known NAD-glycohydrolase activity of this enzyme (14). The present paper deals with kinetic analyses and interpretation of these phenomena.

EXPERIMENTAL PROCEDURES

Calf thymus poly(ADP-ribose) polymerase was purified to homogeneity by a published method and coenzymic DNA was isolated simultaneously (cf. 15). Partially purified rat liver poly(ADP-ribose) polymerase was isolated to stage IV as reported (10). ADP-ribose, NAD and poly(ADP-ribose) were identi-

fied by high performance liquid chromatography (16a, b). Gel electrophoresis and enzymatic assays and the isolation of rat liver nuclei were carried out as published (17). Rapid isolation of ADP-ribosylated proteins by Sephadex G-50 column centrifugation was performed as described (18).

HPLC identification of nucleotide products from neutral hydroxylaminolysis and base hydrolysis of mono-ADP-ribosylated nuclear proteins was carried out as follows. Sixty µg of nuclei were incubated with 100 nM [^{32}P]-NAD (S.A. ≈ 800 Ci/mmol, NEN) for 10 minutes. The reaction was stopped by addition of 1 ml of 20% TCA followed by centrifugation (2000xg). The pellet was washed further with 3 additional portions of 20% TCA, until the supernatant did not contain any appreciable radioactivity, and then twice with 1.5 ml portions of diethyl ether to remove TCA and lipids. The pellet was then digested either with neutral hydroxylamine (1 M at 37°C for 1 hour) or with aqueous potassium hydroxide (1 M at 56°C for 1 hour). Samples were acidified to pH 5.0, centrifuged, and the clear supernatant injected into the HPLC system. The HPLC conditions were the same as described previously (16a) except the flow rate was 1.50 ml/min.

Rat liver nuclei (protein content 10 mg/ml) were extracted with an equal volume of 0.5 M KCl, 50 mM Hepes, 5 mM DTT pH 7.2 by incubation for 30 minutes at 4°C, then centrifuged at 105000xg for 30 minutes at 4°C. The supernatant (2.7 mg/ml protein content) was used for further experiments. To 200 µl nuclear extract, 60 µl of 750 mM Tris HCl, pH 8.0, 50 mM $MgCl_2$, 10 mM DTT and [^{32}P]-NAD were added (total volume 300 µl) and incubated for 10 minutes at 23°C. The final NAD concentration was 100 nM . Thereafter 250 µl of the sample was injected into a TSK W-2000 HPLC column and chromatographed with 25 mM Hepes, pH 7.4, 0.25 M KCl, 2.5 mM DTT elution buffer at a 0.5 ml/min. flow rate at 23°C. Half-ml

aliquots were collected and 50 µl of each fraction was tested for radioactivity. Fractions 34-35 containing the adduct were collected and used for experiments shown in Figure 2B. Alternatively 5×10^{-8}M purified calf thymus poly(ADP-ribose) polymerase was incubated with 100 nM [^{32}P]-NAD for 20 minutes at 23°C under the conditions described in the Legend to Figure 1. The total volume was 100 µl. At the end of incubation, the macromolecules were separated from the unreacted [^{32}P]-NAD by Sephadex G-50 column centrifugation (18) using 10.0 mM Tris HCl pH 7.4, 10 mM $MgCl_2$, and 2.0 mM DTT as equilibration buffer. Ninety to 95% ADP-ribose was bound to proteins. This was determined by hydrolysis of samples with 1 N NaOH for one hour at 23°C, followed by neutralization and HPLC analyses (16a) of products. In addition, products were also identified by PEI-cellulose TLC, developed with 0.9 M acetic acid, 0.3 M LiCl running buffer.

RESULTS

Automodification of purified poly(ADP-ribose) polymerase of calf thymus was determined at nM concentrations of NAD. Initial velocities were plotted against NAD concentrations as shown in Figure 1. At nM concentrations (lower curve) a concave curve was obtained, suggesting multiple binding sites for NAD. On the other hand, when v_{init} is plotted against µM concentrations of NAD - a practice generally followed - no anomalous relationship between v_{init} and NAD concentrations could be detected (Figure 1, upper curve).

The nature of the ADP-ribose enzyme adduct was studied with an extract of rat liver nuclei and also with purified enzymes, keeping NAD concentrations at or below 100 nM. It is of interest that regardless of the purification stage of poly(ADP-ribose) polymerase or the time of incubation with 100 nM or lower concentration of NAD mono-ADP-ribose protein adducts represented 85-90% of protein products. With crude nuclear extracts, depending on the time of incuba-

tion, mono-ADP-ribosylated histones were also formed, apparently as the result of transesterification from enzyme-ADP-ribose (Bauer, P.I. and Kun, E., unpublished). Rat liver nuclei were extracted with 0.5 M KCl and this extract incubated with 100 nM NAD, and the ADP-ribosylated enzyme was isolated on a TSK W-2000 HPLC column as shown in Figure 2A. An identical peak was obtained when the homogenous calf thymus enzyme was ADP-ribosylated. The same ADP-ribose-enzyme adduct was also isolated by Sephadex centrifugation at 4°C (cf. 18) in order to maintain maximal enzymatic activity suitable for the identification of the pH stability of the ADP-ribose enzyme bond (Figure 2B). It is evident that this bond was very sensitive to OH^- and significant hydrolysis occurred above pH 7.0. The nature of the hydrolysis product was determined by HPLC (cf. 16 a.,b.). Hydrolysis by hydroxylamine (cf. 20) at pH 7.0 or hydrolysis at pH 8.0, yielded only ADP-ribose and at pH 14 ADP-ribose was cleaved to AMP + ribose-phosphate as shown by results from Hilz's laboratory (cf. 1).

The kinetics of synthesis and hydrolysis of ADP-ribose-enzyme adducts are illustrated in Figure 3A and B. Partially purified rat liver poly(ADP-ribose) polymerase (Figure 3A) catalyzed the mono-ADP-ribosylation of the enzyme protein at pH 8.0 following a time course shown in curve 1, experimental points representing acid precipitable ADP-ribose protein adducts. After about 50 minutes the rate declined, indicating an increased hydrolysis of enzyme ADP-ribose adducts. When the forward reaction was inhibited by nicotinamide (or benzamide) at 20 minutes, a rapid decay of adducts was evident (curve 2). Following acid precipitation, products were suspended in 1 M NH_2OH (pH 7.0) and incubated at 37°C for 1 hour. Controls, containing no NH_2OH, only buffer, were treated identically. About 80-90% of acid precipitable materials was hydrolyzed by NH_2OH at pH 7.0 in 1 hour and curve 3 was obtained. Hydroxyl-

aminolysis performed in the inhibited system (curve 2) resulted in curve 4. It was apparent that the NH_2OH sensitive/resistant bond ratio was nearly the same in both control (curve 1) and inhibited (curve 2) systems. In a separate series of experiments, the ADP-ribose enzyme adducts were isolated (Figure 3B) by Sephadex centrifugation (18), as indicated by a downward arrow. Without addition of NAD, the enzyme-ADP-ribose adducts hydrolyzed at pH 8.0 and this rate was not appreciably altered by nicotinamide (or benzamide). Added NAD maintained the steady state level of ADP-ribose enzyme adducts. These experiments which are shown in Figure 3B (inset) were done with the homogenous calf thymus polymerase. The sum of results (Figure 3A and 3B) demonstrate that the first product formed at 100 nM NAD concentration is an unstable monomeric adduct of ADP-ribose and the enzyme protein and this adduct spontaneously decomposes at pH 8.0 in the absence of NAD that supports the forward reaction. The same enzyme-mono-ADP-ribose adduct is formed in crude and purified enzyme preparations. It is noteworthy that even prolonged incubation with below 100 nM NAD only yields monomers with only traces of short oligomers, clearly discriminating this initiation step from elongation, that requires higher than 100 nM NAD as substrate. This was verified by previously developed techniques (16a,b) tested within 10 to 100 minutes following enzymatic ADP-ribosylations (Figure 3). The bond between ADP-ribose and protein was identified as an NH_2OH sensitive ester (first order rate constant of hydrolysis by NH_2OH at pH 7.0, 23°C is $K^I = 1.04\ h^{-1}$). Analyses of products formed at pH 8.0 or at pH 7.0 in the presence of NH_2OH from enzyme-bound ADP-ribose yielded only ADP-ribose and no NAD was generated from added nicotinamide.

With the aid of NAD analogs and inhibitors, it was possible to discriminate kinetically between the initiation and elongation catalysis performed by the homogenous calf thymus enzyme. Initiation was determined at nM concentration and elongation at 9 μM NAD, (Table , Experiment 1). Formation of polymers at 9 μM NAD was ascertained by HPLC (16a,b). None of the NAD analogs had an appreciable effect on elongation, but at nM concentrations the initiation reaction was notably activated, an effect that was especially pronounced at 12 nM with acetyl-pyridine-NAD (Experiment 3, 310%), and 12 nM nicotinic acid NAD (Experiment 5, 475%). Different effects on initiation and on elongation were obtained with nM concentrations of benzamide and its amino analogs, which are known inhibitors of the enzyme (19). As shown in Experiments 7 to 10, initiation determined at 25 nM NAD concentration is activated by 263% by 30 nM benzamide and by 225% by 12 nM 3-aminobenzamide. On the other hand elongation, that was determined at NAD concentrations varied between 0.01 - 1.0 mM and benzamide concentrations varied between 5 to 200 μM, exhibited competitive inhibition with benzamides with varying K_i values. Our K_i values differ from those originally reported with crude nuclear extracts as a source of polymerase (cf. 19). This discrepancy is probably due to the experimental conditions since we applied homogenous calf thymus enzyme and saturating concentrations of coenzymic DNA (15). Due to the complication introduced by qualitatively different responses on initiation and elongation to different concentrations of an inhibitor (e.g., 3-aminobenzamide), the effects of these substances on the polymerase can be highly variable and activation or inhibition may be obtained as a function of NAD concentration.

Mono-ADP-ribosylation of proteins with 100 nM NAD as substrate can be readily demonstrated also with isolated rat liver nuclei (cf. 13). The quantity

of proteins isolated by gel electrophoresis (17) was determined by densitometry using the purified enzyme protein and histones as standard. Specific radioactivity was determined for each histone species (cf. 17). There was a marked difference in the specific activities (i.e., mol of ADP-ribose/mol protein) of the enzyme (calculated from Figure 1) and histones: (0.05 - 0.1 mol ADP-ribose per mol enzyme and 0.002 - 0.004 mol ADP-ribose per mol histone H_1), clearly indicating the predominance of auto-mono-ADP-ribosylation of the enzyme protein even in nuclei.

DISCUSSION

Our experimental results are consistent with a mechanism that includes the formation of an unstable mono-ADP-ribose enzyme protein initiator adduct that is likely to be the first catalytic product of poly ADP-ribosylation. Recognition of this initiator adduct (11,12) has been made difficult in the past by the use of μM NAD concentrations, which support both initiation and elongation. Identification of the initiator mono-ADP-ribose adduct depends on nM concentrations of NAD as substrate.

By accepted criteria of hydroxylaminolysis, developed in Hilz's laboratory (cf. 1) the ADP-ribose protein bonds are identified as unstable ester bonds, in agreement with the bonds described for poly(ADP-ribose) protein adducts (cf. 1-4) except we determined hydroxylaminolysis at pH 7.0 whereas others (20) at pH 7.5. We find that the mono-ADP-ribose enzyme adducts are in a dynamic state with respect to NAD even at physiological pH values and there is a constant flow of OH^- catalyzed release of ADP-ribose from NAD at the ADP-ribose enzyme initiator template. This "apparent NAD glycohydrolase" activity at the mono-ADP-ribose binding site can provide ADP-ribose for Schiff base formation (6,21) with the enzyme protein or with proteins that are located near the initiation

site. We have shown that the hydroxylamine insensitive ADP-ribose-protein adducts, comprising 15-30% of total adducts, correspond to Schiff bases as identified by selective borotritiation (6) (Bauer, P.I. and Kun, E., results presented elsewhere).

Rates of mono-ADP-ribosylation of the enzyme protein are greatly increased by NAD homologs which have no effect on polymerization (see Table). Details of the abnormal kinetics at nM NAD concentrations and the role of modifiers are subject to further studies. The large effect of desamino NAD as a "forward" activator of initiation is probably physiologically significant, since this substance is a known metabolic precursor of NAD. This novel, probably allosteric effector phenomenon on poly ADP-ribosylation initiation, which is predictably regulated by a probably localized decrease of NAD concentration at chromatin sites to nM levels, may explain the 180 fold prevalance of monomer adducts over polymer-protein compounds, as found experimentally in AH7974 cells (20). Another feature of the unstable mono-ADP-ribose enzyme initiation adducts is their propensity to serve as ester-donors for ADP-ribose transesterification reactions to certain glutamate and lysine carboxyl endgroups of histones as has been demonstrated (22,23) In agreement with Kreimeyer, et al. (20) on a molar basis ADP-ribosylation of histones appears to be minimal as compared to the enzyme protein. The large quantities of nuclear histones containing low molar specific activity of ADP-ribose (i.e., mol ADP-ribose/mol histone) will necessarily exhibit large spots of auto-radiograms. Similar transesterification mechanisms may account for ADP-ribosylation of topoisomerase (24). It has been reported (25) that enzymatic cleavage of histone-ADP-ribose adducts can be catalyzed by a cytosolic enzyme, resulting in a rearranged product of ADP-ribose: ADP-3"-deoxypentose-2"-ulose, that can be chromatographically distinguished from ADP-ribose. However, hydroxyl-

aminolysis at pH 7.0 of the same adduct yields only ADP-ribose (25). Since no other substance except ADP-ribose was identified as a product of hydroxylaminolysis of the ADP-ribose enzyme initiator adduct, there seems to be no complicating interference by the reported ADP-ribosyl-protein lyase in our system. Actually this enzymatic reaction has been rigorously demonstrated to act on ADP-ribose adducts of histone H_1 and H_2B (cf. 25).

Demonstration of an unstable ADP-ribose-enzyme initiation adduct, detectable at nM NAD concentrations and possessing special regulatory properties (e.g., forward activation by desamino NAD) provides a catalytically feasible model for initiation and limited trans-ADP-ribosylation and also explains the hitherto enigmatic "extra"-NAD-glycohydrolase activity of poly(ADP-ribose) polymerase.

Acknowledgments

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LEGENDS

Figure 1

Upper Curve: 5.2×10^{-9} M enzyme was incubated with [^{14}C]-NAD (0-200 µM concentration range) in the presence of 6 µg/ml coenzymic DNA for one minute at 23°C and products assayed as reported (17). The volume of the incubation mixture was 100 µl. The specific activity of nicotinamide [U-^{14}C]-adenine dinucleotide (Amersham) was 300 mCi/mmol.

Lower Curve: 2×10^{-9} M enzyme was incubated with [^{32}P]-NAD (0-100 nM concentration range) for one minute at 23°C in the presence of 6 µg/ml coenzymic DNA, in a total volume of 30 µl.

Figure 2

A. Isolation of enzyme-bound ADP-ribose by HPLC.

B. The dependence of hydrolysis of ADP-ribose enzyme adduct on pH. Identical results were obtained with crude and purified enzymes.

Figure 3

A. Effect of nicotinamide and hydroxylamine on products synthesized by partially purified rat liver poly(ADP-ribose) polymerase (step 4 of ref. 10). Twenty µg enzyme protein was incubated with 100 nM [^{32}P]-NAD and 23°C, and aliquots were removed at specified times. ADP-ribose enzyme adducts were determined by 10% TCA precipitation.

Curve 1 = without additions.

Curve 2 = addition of 160 mM nicotinamide or 10 mM benzamide.

Curve 3 = hydroxylaminolysis of experiment described in Curve 1.

Curve 4 = hydroxylaminolysis following addition of nicotinamide or benzamide.

B. (Inset) 4.5×10^{-8} M purified calf thymus enzyme was incubated with 50 nM of [^{32}P]-NAD in a total volume of 150 µl under the conditions described in Figure 3. Five µl aliquots were withdrawn and the 10% TCA precipitable radioactivity determined. Beginning at 20 minutes aliquots of ADP-ribosylated enzyme were withdrawn and NH_2OH sensitivity of ADP-ribose protein bonds were assayed at pH 7.0 (—Δ—Δ—, —o—o—, see Results).

Legend To Table I

Enzymatic activities were determined under conditions described in Legend to Figure 1. NAD-analogs and their concentrations are given in column 1, NAD concentrations in column 2, v_{init} of mono-ADP-ribosylation in column 3, v_{init} of elongation in column 5. In Experiment No. 1, elongation rates and initiation rates are given (at 9 µm and 25 nM NAD concentrations respectively) in the absence of NAD analogs, and column nos. 4 and 6 expresses % activities of systems containing NAD analogs.

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TABLE I. Effects of NAD analogs on the initiation and elongation rates of the automodification of purified calf thymus poly(ADP-ribose) polymerase.

No.	Analog	Substrate NAD	Initiation Rate fmol ADP-ribose/ pmol enzyme in 1 min.	% of No. 1	Elongation Rate pmol ADP ribose/pmol enzyme in 1 min.	% of No. 1
1	-	9 μM	-	-	6.0	100
	-	25 nM	10.5	100	-	-
2	Quanino-NAD					
	12 nM	25 nM	13.0	125	-	-
	30 nM	25 nM	16.0	160	-	-
	1 mM	9 μM	-	-	5.6	93
3	Acetyl Pyridine-NAD					
	12 nM	25 nM	32.3	310	-	-
	30 nM	25 nM	19.3	190	-	-
	1 mM	9 μM	-	-	7.0	116
4	Aldehyde Pyridine-NAD					
	12 nM	25 nM	17.8	171	-	-
	30 nM	25 nM	15.6	149	-	-
	1 mM	9 μM	-	-	6.5	110
5	Nicotinic Acid-NAD					
	12 nM	25 nM	49.4	474	-	-
	30 nM	25 nM	28.3	270	-	-
	1 mM	9 μM	-	-	7.2	120
6	Amino Pyridine-NAD					
	12 nM	25 nM	30.7	293	-	-
	30 nM	25 nM	28.2	269	-	-
	1 mM	9 μM	-	-	6.85	110
7	Benzamide					
	12 nM	25 nM	11.5	110		
	30 nM	25 nM	25.4	263		
	5-200 μM	0.01-1 mM	-	-	K_i = 39 μM	
8	2-Aminobenzamide					
	12 nM	25 nM	8.6	82		
	30 nM	25 nM	10.6	100		
	5-200 μM	0.01-1 mM	-	-	K_i = 71 μM	
9	3-Aminobenzamide					
	12 nM	25 nM	23.5	225		
	30 nM	25 nM	15.8	150		
	5-200 μM	0.01-1 mM	-	-	K_i = 12 μM	
10	4-Aminobenzamide					
	12 nM	25 nM	13.2	120		

Fig 1.

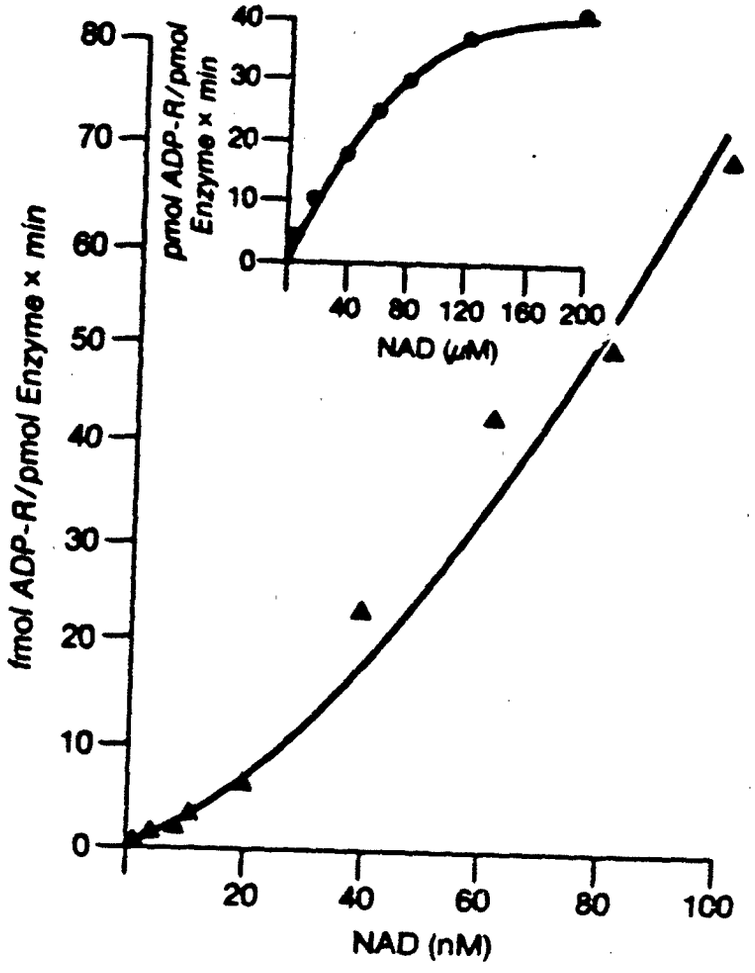

fmol ADP-R/pmol Enzyme × min
80
70
60
50
40
30
20
10
0
20
40
60
80
100
NAD (nM)
pmol ADP-R/pmol Enzyme × min
40
30
20
10
0
40
80
120
160
200
NAD (μM)

Fig 2.

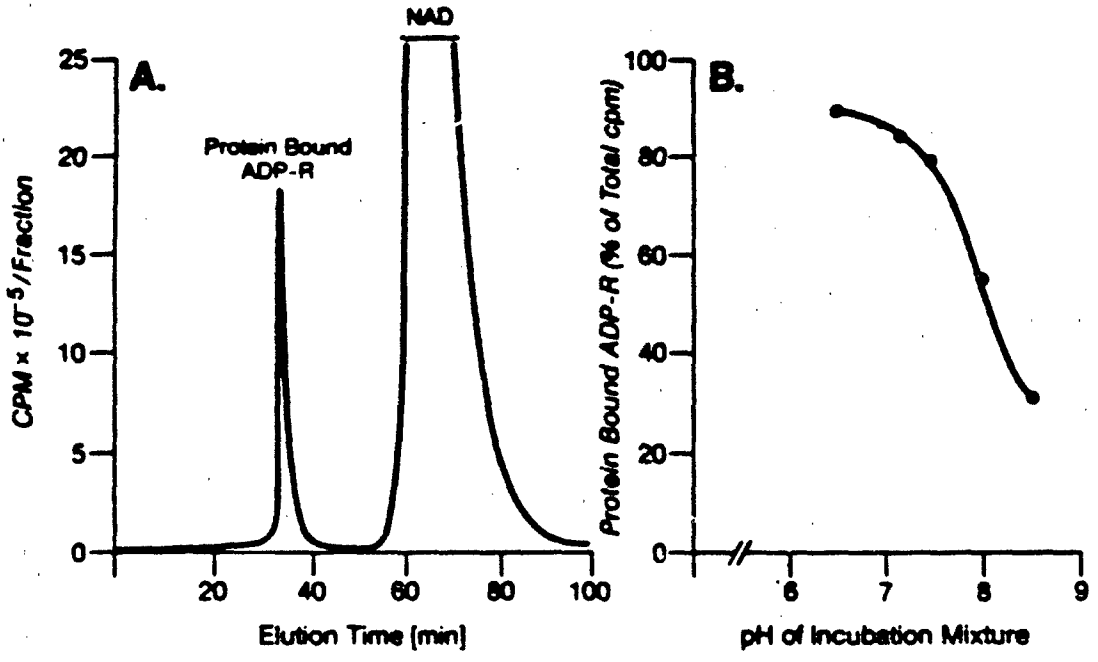
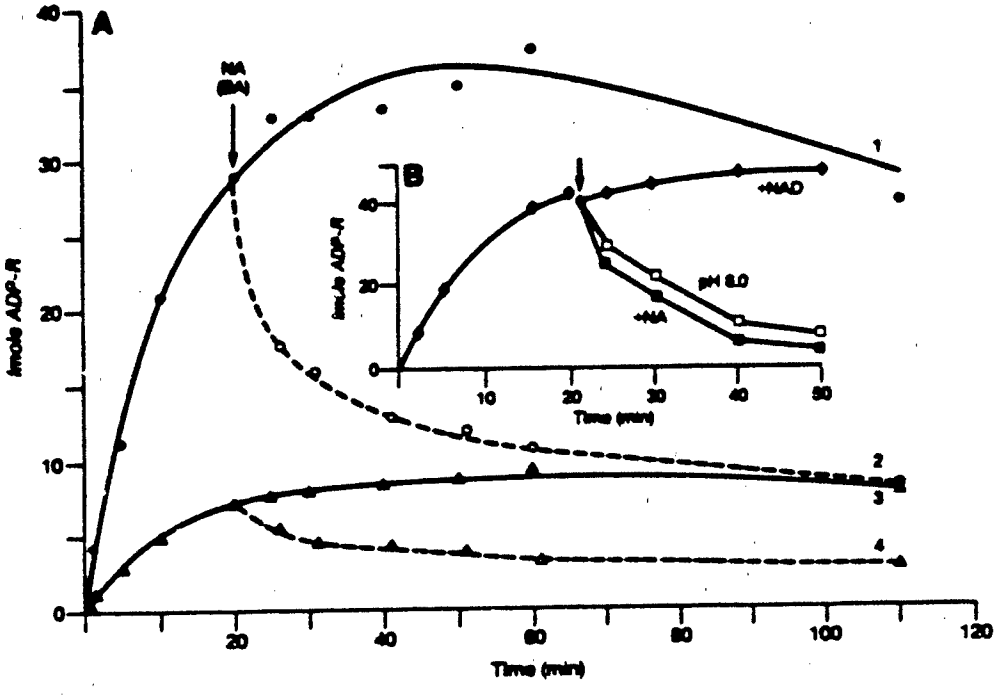

A.
NAD
Protein Bound
ADP-R
CPM x 10⁻⁵/Fraction
25
20
15
10
5
0
20
40
60
80
100
Elution Time (min)
B.
Protein Bound ADP-R (% of Total cpm)
100
80
60
40
20
0
6
7
8
9
pH of Incubation Mixture

Fig. 3.



3

MECHANISM OF INACTIVATION OF POLY(ADP-RIBOSE) POLYMERASE OF RAT LIVER NUCLEI BY 4-DIAZO-BENZAMIDE. COVALENT BINDING OF THE DIAZONIUM COMPOUND TO DEOXYGUANINE OF COENZYMIC DNA.

No 3.

Abstract

The 4-diazonium derivative of 4-amino-benzamide was prepared and conditions for the inactivation of nuclear poly(ADP-ribose) polymerase by this molecule established. Labelling with [^{3}H] in the 3 and 5 position was done by I $\longrightarrow$ [^{3}H] exchange in 3,5-diiodo-4-amino-benzamide which was then diazotized, yielding neat isotope incorporation into 4-diazo-benzamide. The nuclear enzyme was inactivated by treatment (at 4°C) with 4-diazo-benzamide, whereas 4-diazo-benzoic acid was ineffective. Benzamide, which is a reversible inhibitor of poly(ADP-ribose) polymerase, prevented enzyme inactivation by 4-diazo-benzamide. With the aid of 3,5[^{3}H]-4-diazo-benzamide and 3,5[^{3}H]-4-diazo-benzoic acid it was shown that the diazonium compounds up to 5 mM selectively bind to dGMP of DNA but not to proteins. The catalytic function of coenzymic DNA was abolished by 4-diazo-benzamide parallel to its covalent addition to dGMP. 4-diazo-benzoic acid, although binding covalently to coenzymic DNA did not interfere with its catalytic activity. It is concluded that the covalent adduct of the benzamide residue of 4-diazo-benzamide to dGMP of coenzymic DNA is kept at the inhibitory site of the enzyme by the tight binding of DNA to its binding domain on the enzyme protein. Therefore, the inhibition kinetics of DNA bound benzamide residue appears to be inactivation. The molecular vicinity of catalytic and DNA binding sites on the enzyme protein appears probable.

A unique feature of nuclear poly(ADP-ribose) polymerase (E.C. 2.4.99) is its dependence for catalytic activity on DNA (cf. 1,2). This DNA can be simultaneously isolated with the enzyme protein as a species (or sequence) that remains tightly associated with the enzyme protein during purification and may be separated by hydroxylapatite chromatography (3). The precise identity and catalytic function of this "coenzymic" (cf. 3) DNA in the mechanism of poly-ADP-ribosylation constitute as yet unresolved problems. It was shown that enzymatic cleavage or nicking of double-stranded circular DNA produces coenzymatically active DNA for poly(ADP-ribose) polymerase (4,5,6,7) and synthetic oligodeoxynucleotides can kinetically replace coenzymic DNA in vitro, (8,9). However, it is as yet unclear to what extent these model studies relate to in vivo conditions where it may be assumed that a specific binding of a specific DNA species or sequence to the enzyme protein represents the regulatory DNA coenzyme of the polymerase (10 a., b.)

If a DNA species or sequence is to participate in the molecular mechanism of poly-ADP-ribosylation, it would be expected that a correlation should exist between DNA binding sites and the catalytic sites of the enzyme protein. A dependence of K_m values for NAD on the concentration of coenzymic DNA (11) appears to support this postulate. We have provided evidence that identifies lysine residues as hydrophobic binding sites for coenzymic DNA (12,13). Kinetically identifiable catalytic sites of the enzyme are the nicotinamide, or NAD binding sites, which are readily recognizable by enzyme inhibition with benzamides (14), known competitors of NAD. We assumed that the acid amide moiety of benzamide could serve as an active site directing group and appropriate substituents attached to

the benzene ring may form covalent bonds with amino acid residues in the vicinity of the polymerase enzyme site. The diazonium derivative of 4-amino-benzamide could predictably react with tyrosine, histidine or lysine residues (15) and by isolation of these adducts the theoretical possibility exists to identify neighboring peptide structures to a presumed active site of the enzyme. Following this strategy, we found with the aid of highly tritiated 4-diazo-benzamide, that contrary to predictions, this molecule between 1 to 5 mM did not bind covalently to the enzyme protein but reacted preferentially with deoxyguanine of the purified coenzymic DNA, or with dGMP of DNA present in liver nuclei. Since the covalent binding of 4-diazo-benzamide to DNA resulted in apparent enzyme inactivation, an effect not shared by the covalent binding to DNA of 4-diazo-benzoic acid, it was concluded that the inhibitory acid amide group was kept in place at the nicotinamide binding site of the enzyme by the tight association of the benzamide residue-containing coenzymic DNA with the enzyme protein. The present paper is concerned with the mechanism of inactivation of poly(ADP-ribose) polymerase of liver nuclei by 4-diazo-benzamide. The site of binding of 4-diazo-benzamide was determined both in a reconstructed system, composed of purified polymerase protein and coenzymic DNA, and in liver nuclei.

EXPERIMENTAL PROCEDURES

1. Synthesis of [^{3}H]-4-amino-benzamide. This synthesis was carried out in two steps: (a) preparation of 3,5-diiodo-4-amino-benzamide; (b) replacement of I by [^{3}H]. (a) 3,5-diiodo-4-amino-benzoic acid was dissolved in absolute dioxane at a concentration of 50 mM. N-hydroxysuccinimide (100 mM) and dicyclohexylcarbodiimide (80 mM) were added. The volume of the incubation mixture was 50 ml. After incubation for two hours at room

temperature, 50 ml of 2.9% ammonium hydroxide was added and incubation continued overnight. The reaction mixture was rotoevaporated twice from H_2O and twice from methanol. Finally it was suspended in 25 ml of water and filtered on a G3 sintered glass filter. The solid material was washed with H_2O, ether, then extracted with 3 x 15 ml of dioxane followed by rotoevaporation and the product was extracted with 50% MetOH. Final purification was achieved by HPLC on an Ultrasphere ODS (Beckman-Altex) reversed-phase column with isocratic elution (50% MeOH-H_2O). The product eluted with a retention time of 12 minutes and its purity was further tested on TLC/silica gel with $CHCl_3$, EtOH or EtAc as developing solvents. Final yield was 12.4%. The high resolution mass spectrum displayed the expected molecular ion, m/e calculated for $C_7H_6I_2N_2O$: 387.8569; found 387.8538 (deviation = 8.0 ppm).

(b) Labelling of 3,5-diiodo-4-amino-benzamide (with [^{3}H]) by the I $\longrightarrow$ [^{3}H] exchange reaction. One to two milligrams of the iodo compound were dissolved in 100 to 300 µl of methanol and the solution was applied to 10 to 15 pellets (1/8") of silica-alumina catalyst support (#980-25, Davison Chemical Division, W.R. Grace & Co., Baltimore, Md.) or to Ni catalyst (5%) prepared according to Cao and Peng (16). The impregnated pellets were dried overnight in vacuo. The dry pellets were then placed on the cold finger in a tritiation apparatus described earlier (17). The apparatus was evacuated to less than 0.13 Pa and tritium gas admitted to 400-667 Pa (or 3-5 torr) before adding liquid nitrogen to the cold finger and initiating the microwave discharge with Tesla coil. The microwave power input was 80 or 20 watts for 400 to 667 Pa of tritium gas pressure respectively. After 5 minutes irradiation excess tritium gas was removed

and the apparatus flushed twice with helium before opening to retrieve the pellets. Under these conditions, activated tritium species from the plasma can replace the I atom in the C-I bond of the iodinated molecules with T to produce carrier-free specifically tritium-labelled amino-benzamides or acids (18). The tritiated sample was evaporated from methanol to remove the labile tritium. The product was extracted from the solid support with 2 x 1 ml of methanol and applied onto preparative silica gel TLC, developed with ethylacetate. The position of 4-amino-benzamide was identified with non-radioactive UV markers. The 3,5-[^{3}H]-4-amino-benzamide was eluted from the silica gel powder with methanol. Finally it was purified by reversed-phase HPLC, using an Ultrasphere ODS (5μ, 25 mm x 4.6 mm ID) column. The sample was dissolved in water and injected into the water-equilibrated column. After washing the column with H_2O for 10 minutes (flow rate 1 ml/min.) the 3,5-di[^{3}H]-4-amino-benzamide was eluted with 25% MetOH as isocratic eluant (RT 19.5 min.). The specific activity of the compound was 14.4 Ci per mmol and 25.2% of injected radioactivity was recovered as the purified product.

2. Preparation of 4-diazo-benzamide (20 mM final concentration). Fifty-five mg of 4-amino-benzamide was dissolved in 20 ml of 0.15 M HCl and incubated for 20 minutes at 4°C with 50 mg of $NaNO_2$ added as solid and stirred. After incubation, the pH was set to 5.0 - 5.5 with 3 M NaOH. Under these conditions, the total amount (98-100%) of 4-amino-benzamide was converted to the diazo form. This was determined by reversed-phase HPLC as described above (RT = 9.5 min.). Absorption spectra are shown in Figure 1, illustrating alos the stability of 4-diazo-benzamide (see also No. 1 in Figure 5).

3. Preparation of [^{3}H]-4-diazo-benzamide. Solutions of one to ten µCi of 3,5-[^{3}H]-4-amino-benzamide and various amounts of non-radioactive 4-amino-benzamide, to achieve desired specific activity, were dried under N_2 stream into Eppendorf centrifuge tubes and the dried material dissolved in 20 µl of 0.15 M HCl. Two µl of a solution of 2.5 mg/ml $NaNO_2$ were added and incubated for five minutes. The pH was adjusted between 5.5 - 6.0 upon addition of an equal volume of 250 mM Hepes buffer pH 7.4. [^{3}H]-4-diazo-benzoic acid was prepared in the same way.

4. Calf thymus poly(ADP-ribose) polymerase was purified to homogeneity by a published method (3) and coenzymic DNA separated by hydroxylapatite chromatography (cf. 3). Isolation of rat liver nuclei, assays for poly(ADP-ribose) polymerase, isolation of macromolecular products by gel electrophoresis and isolation of nucleotides and the dGMP adduct were done by methods reported earlier (19,20,21,22).

RESULTS

Figure 1 Here

Since 4-diazo-benzamide would be expected to be unstable, it was essential to establish predictable in vitro conditions, suitable for enzyme inhibition assays. When the absorbance of freshly prepared 4-diazo-benzamide (see Experimental Procedures) was compared with 4-amino-benzamide, distinct spectra were obtained (Figure 1A) and at pH 7.4 at room temperature a plot of absorbance changes vs. time (Figure 1B) illustrates the decay rate of the diazo compound. From the rate of change in absorbance (at 270 nm) the pH dependence of decay was also determined as illustrated in the inset of Figure 1B. At ice bath temperature the rate of decay of 4-diazo-benzamide was followed up to 60 min. at pH 7.4 (see Figure 5, No. 1). Studies

concerned with enzyme inactivation by 4-diazo-benzamide and adduct formation were done under these conditions. Particle-bound poly(ADP-ribose) polymerase, present in liver nuclei are best suited for the determination of the mode of action of 4-diazo-benzamide because nuclei can be readily reisolated after treatment with 4-diazo-benzamide and degradation products removed by centrifugal washings. At varying concentrations of 4-diazo-benzamide, liver nuclei were incubated in an ice bath for varying lengths of time. The effect of 4-diazo-benzamide on the nuclear system was tested by assaying poly(ADP-ribose) polymerase activity (19). The reaction of 4-diazo-benzamide was terminated by addition of an excess of chloroacetyl tyrosine, sufficient to bind the unreacted diazonium compound. As shown in Figure 2, poly(ADP-ribose) polymerase was inactivated corresponding to a first order rate, determined between 0 and 2.5 mM concentration of 4-diazo-benzamide (Figure 2A) and a plot of K^I against the concentration of 4-diazo-benzamide gave a linear correlation on a semilogarithmic scale (Figure 2B).

Figure 2 Here

The nature of the inactivation of poly(ADP-ribose) polymerase of nuclei by 4-diazo-benzamide was tested by the prevention of inactivation by benzamide which is a known reversible inhibitor of the enzyme. The effect of 4-diazo-benzoic acid, that was prepared exactly as 4-diazo-benzamide, was also determined. These results are summarized in Table I. In Experiment No. 2, nuclei were exposed to the diazotizing reagent, enzymatic activity being practically identical with controls (No. 1). Benzamide at 7.7 mM completely inhibited poly(ADP-ribose) polymerase (not shown) but this inhibition was completely reversed by removal of benzamide by centrifugal washing of nuclei (Experiment 3). Pre-incubation of nuclei

Table I Here

with 1.6 mM 4-diazo-benzamide as described in Figure 2, resulted in significant enzyme inactivation which could not be reversed by centrifugal washing of nuclei (Experiment 4). Simultaneous exposure of nuclei to 2.3 and 7.7 mM benzamide and 1.6 mM 4-diazo-benzamide resulted in a benzamide concentration-dependent prevention of inactivation of the polymerase by 4-diazo-benzamide, indicating a competition of benzamide with the macromolecularly-bound benzamide residue of 4-diazo-benzamide. It is apparent that the reversible inhibitor benzamide by binding to the acid amide recognizing site of the enzyme displaces the covalently-bound benzamide residue of 4-diazo-benzamide, and after centrifugal washing that removes benzamide catalytic activity is restored. These results also suggest that the covalently-bound benzamide residue previously displaced by the free benzamide does not find its way back to the catalytic site on the enzyme after removal of free benzamide. When 1.6 mM 4-diazo-benzoic acid was exposed to nuclei exactly as had been done with 4-diazo-benzamide, no appreciable inactivation of poly(ADP-ribose) polymerase could be observed (Experiment 7). In fact, the activity of the enzyme was the same as in the benzamide-protected system (compare 6 and 7). These results show that the apparent inactivation of the poly(ADP-ribose) polymerase system is attributable to the acid-amide moiety of the covalently-bound residue of 4-diazo-benzamide and the predictable covalent binding of the diazonium compound of benzoic acid to a component of the system per se has no inhibitory effect.

It was tested in separate experiments by the isolation with HPLC of non-radioactive benzamide following incubation with [^{3}H]-4-diazo-benzamide (not shown), that 4-diazo-benzamide did not react with benzamide under given

conditions. Therefore this potential artefact, which could mimick a protective effect of benzamide, was ruled out.

In subsequent experiments we determined the macromolecular binding site of 4-diazo-benzamide by isolating both protein and DNA components of the poly(ADP-ribose) polymerase system with the aid of specific labelling probes: [^{3}H]-4-diazo-benzamide and [^{32}P]-ADP-ribose derived from NAD. Two systems were studied. First the purified system composed of the polymerase enzyme, coenzymic DNA and histones; second the particle-bound nuclear system. It should be noted that in these studies we were only concerned with the binding of either [^{3}H]-4-diazo-benzamide or [^{32}P]-NAD derived ADP-ribose to macromolecular components, therefore each ligand was incubated with the systems separately under specific conditions. Simultaneous exposure to both 4-diazo-benzamide and NAD results in the inhibition of ADP-ribosylation as described above. In a test consisting of the purified enzyme protein, coenzymic DNA and added histones results shown in Figure 3 were obtained. Protein and DNA components were isolated by gel electrophoresis as reported earlier (19,20). Labelling with [^{3}H]-4-diazo-benzamide of the purified enzyme protein together with coenzymic DNA at ice bath temperature was followed by gel separation of DNA and proteins. In a parallel run the enzyme + coenzymic DNA were incubated with [^{32}P]-labelled NAD at 25°C and ADP-ribose protein adducts were also isolated by gel electrophoresis (19). In this gel system, DNA did not enter the gel and was recovered in the first slices on top of the gel. As demonstrated in Figure 3 [^{3}H] labelled adduct of 4-diazo-benzamide appeared exclusively in the coenzymic DNA component of the poly (ADP-ribose) polymerase system that was reconstructed from purified enzyme protein, coenzymic DNA and histones. On the other hand, ADP-ribosylation

Figure 3 Here

occurred predominantly on the enzyme protein (120 kDa) and to a lesser extent on histones. The calculated specific activity of DNA, representing mols of p-4-diazo-benzamide residue was 200 pmol/μmol base, i.e., 1 out of 5,000 bases was labelled. In the system composed of the purified enzyme and coenzymic DNA the presence of unknown protein components is minimized. Therefore electrophoretic separation of components as shown in Figure 3 indicating [^{3}H]-4-diazo-benzamide to be associated with DNA provides reasonable evidence of the selectivity of the diazonium salt towards DNA. However, raising the concentration of 4-diazo-benzamide above 5 mM (10, 20 mM) results in progressive non-specific labelling of protein components also, thus selectivity is lost. For these reasons, the reproducibility of our results depends on the low concentration (up to 5 mM) of 4-diazo-benzamide indicating an apparent higher affinity of 4-diazo-benzamide towards DNA than towards proteins.

As shown in Figure 4A following incubation of nuclei with [^{32}P]-NAD, a number of ADP-ribose acceptors could be isolated by gel electrophoresis, including the enzyme protein, its probable degradation products and histones, exhibiting labelling with ADP-ribose. The abundance of histones in nuclei explains their relatively high labelling (20). Following pre-incubation of nuclei with [^{3}H]-4-diazo-benzamide under identical conditions described for experiments shown in Table I, gel electrophoresis revealed no [^{3}H] label in proteins, only in DNA (Figure 4B). Digestion with nucleases significantly reduced [^{3}H] label in DNA, the residual label probably representing tight DNA-protein complexes not accessible to nucleases (Figure 4C).

Figure 4 Here

The nature of the reaction products between 4-diazo-benzamide and DNA was determined by isolation of a specific deoxynucleotide diazo-benzamide adduct (Figure 5). The separation of spontaneous degradation products of 4-diazo-benzamide by HPLC is shown in Figure 5, Experiment 1, benzamide being

the main product. This degradation took place during prolonged handling of samples and HPLC analyses of 4-diazo-benzamide-DNA adducts, whereas freshly prepared diazonium adducts at 4°C revealed a smaller degree (about 50% of that shown in Experiment 1) of decomposition (compare with Figure 1). Some degradation occurred in all systems where [^{3}H]-diazo-benzamide was incubated either with nuclei (Experiment 2) or coenzymic DNA (Experiment 5) or deoxyguanine monophosphate (Experiments 4 and 5) and in the HPLC histograms, these products are not illustrated. In Experiment 2, four deoxynucleotide monophosphate markers were located by their absorption spectra indicated by downward arrows. In the same experiment (No. 2), deoxynucleotides isolated after the digestion of DNA which has been extracted from nuclei that have been treated with [^{3}H]-diazo-benzamide, were separated by HPLC. No [^{3}H] was found in the region of markers but a new compound distinct from the marker deoxynucleotides appeared that was labelled with [^{3}H]-diazo-benzamide. The same labelled nucleotide was isolated from coenzymic DNA (Experiment 3) and its identity with the adduct with dGMP is illustrated in Experiments 4 and 5. In No. 4, authentic dGMP was reacted with [^{3}H]-diazo-benzamide. The dGMP product was subsequently double-labelled (with [^{14}C]-dGMP and [^{3}H]-diazo-benzamide) as shown in Experiment 5. A mol/mol adduct was identified from the double-labelled product (Experiment 5). It is also apparent that the diazonium derivative of dGMP clearly separates from unreacted [^{14}C]-labelled dGMP. The exact molecular structure of this adduct is under investigation.

Figure 5 Here

It is seen from results shown in Table I that 4-diazo-benzoic acid does not inactivate nuclear poly(ADP-ribose) polymerase, although it was predictable that this diazonium compound can also react with DNA similar to

the inhibitory 4-diazo-benzamide. This was verified experimentally with [^{3}H]-4-diazo-benzoic acid (results not shown). When coenzymic DNA was treated with both diazonium compounds, and coenzymic activity tested with purified poly(ADP-ribose) polymerase comparing equal concentrations of untreated and treated coenzymic DNA, results shown in Figure 6 were obtained. Clearly, only the 4-diazo-benzamide-treated DNA proved inactive as a coenzyme, indicating that the benzamide residue covalently bound to DNA at dGMP sites acts as a highly efficient inhibitor of the enzyme. Non-specific denaturation of DNA by the diazonium compounds was ruled out since 4-diazo-benzoic acid-treated DNA is an equally effective coenzyme as unmodified coenzymic DNA exposed to the same experimental conditions (Figure 6, top curve).

Figure 6 Here

As seen from Figure 6, the binding of [^{3}H]-4-diazo-benzamide to coenzymic DNA and the rate of inhibition of the enzyme exhibited an inverse relationship, whereas the binding of 4-diazo-benzoic acid to DNA had no inhibitory consequences, illustrating that the DNA-bound benzamide residue represents the inhibitory species.

DISCUSSION

At low concentrations of 4-diazo-benzamide apparent inactivation of poly(ADP-ribose) polymerase coincides with a covalent binding of the benzamide residue to dGMP of coenzymic DNA without binding to the protein. Two observations: first, the prevention of inactivation by benzamide and second, the inactivation of coenzymic DNA by 4-diazo-benzamide but not by 4-diazo-benzoic acid are consistent with an "adaptor" role of coenzymic DNA in the mechanism of enzyme inhibition, that kinetically resembles inactivation (Figure 2). The

tight binding of DNA to the enzyme protein thus provides the mechanism to keep the benzamide residue at the enzyme inhibitory site, presumably the NAD-binding site. Whereas 4-amino-benzamide is a relatively weak, reversible competitive inhibitor with a K_i of 75 μM, when the benzamide residue is covalently bound to coenzymic DNA the inhibition is not reversed by centrifugal washings, that is an entirely sufficient method to reverse the inhibitory effect of benzamide itself. This unusual mechanism also implies that the catalytically active enzyme site, the aromatic acid-amide or NAD binding site, is in molecular vicinity to the DNA binding domain of the enzyme, certainly not beyond a distance that is occupied by a benzene ring. Supporting the inference based on kinetics of the vicinity between DNA and NAD sites (cf. 11) our results provide more direct molecular evidence regarding the close positioning of the two binding sites on the enzyme protein. Besides possible further implications in the study of interactions between the two sites - a problem yet to be resolved - our results also predict the feasibility of constructing highly potent antineoplastic drugs that combine DNA-alkylating and poly(ADP-ribose) polymerase inhibitory properties in one molecule. This may be accomplished by replacing the diazo substitution with an alkylating group in benzamide, a synthetic possibility which we are pursuing. The rationale behind this plan is based on observations which demonstrate a significant reinforcement of the anticancer effectivity of DNA-targeted drugs by simultaneous administration of an inhibitor of poly(ADP-ribose) polymerase (23,24,25). It seems predictable that this synergism should be much more pronounced if the same drug molecule can accomplish both DNA-alkylation and inhibition of poly(ADP-ribose) polymerase.

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LEGEND TO TABLE I

Inactivation of nuclear poly(ADP-ribose) polymerase by 4-diazo-benzamide and its prevention by benzamide. Suspensions of rat liver nuclei (6 mg protein, 1.5 mg DNA per 250 µl) was incubated with the diazotizing reagent (Experiment 2) or with 1.6 mM 4-diazo-benzamide (Experiment 4) or 4-diazo-benzoic acid (Experiment 4) at 0 to 4°C for 10 minutes, then nuclei were reisolated and the enzyme was assayed as described in the Legend to Figure 2. In Experiment 3, nuclei were preincubated with 7.7 mM benzamide at 0 to 4°C for 10 minutes then reisolated and benzamide removed by centrifugal washing, and in Experiment 5 and 6, benzamide and 4-diazo-benzamide were present simultaneously during preincubation. Following reisolation of nuclei, poly(ADP-ribose) polymerase was assayed as described (19). These results are a means of 3 experiments with a standard deviation shown in the Table.

LEGEND TO FIGURE 1

A. Absorption spectra of 4-diazo-benzamide and 4-amino-benzamide, 0.1 mM, pH 7.4, at room temperature.

B. Decay of 4-diazo-benzamide at pH 7.4, at room temperature; Inset = dependence of decay rate on pH at room temperature, Coordinate: % of initial absorbance on a log scale; abscissa: time in minutes.

LEGEND TO FIGURE 2

Inactivation of nuclear poly(ADP-ribose) polymerase by 4-diazo-benzamide. Rat liver nuclei (cf. 19) equal to 5 mg protein, were incubated in a volume of 200 µl with different amounts of 4-diazo-benzamide at 4°C. The reaction was stopped by diluting the reaction mixture with 4 ml of 10 mM Tris-HCl pH 7.4, 3 mM $MgCl_2$, 250 mM sucrose, 1 mg/ml bovine serum albumin, buffer containing chloroacetyl tyrosine (1 mg/ml) then centrifuged at 500 x g for 10 min. to reisolate nuclei. The washing process was repeated twice in succession and finally nuclei were resuspended into 150 µl of the same buffer. The nuclear suspension (equivalent to 25-75 µg DNA) were tested for poly(ADP-ribose) polymerase enzyme activity using 9 µM [^{14}C]-NAD (adenine labelled, S.A. = 290 mCi/mmol) as substrate, in 150 mM Tris-HCl pH 8.0, buffer containing 10 mM $MgCl_2$, and 1 mM DTT in a final volume 50 µl, as reported (19).

LEGEND TO FIGURE 3

Binding of 4-diazo-benzamide to coenzymic DNA assayed in the purified reconstructed system.

A. Purified poly(ADP-ribose) polymerase (5 µg), coenzymic DNA (0.2 µg) and total calf thumus histones (10 µg/ml) were incubated in 50 µl incubation buffer (cf. 20) with 100 nM [^{32}P]-labelled NAD (1.1 Ci/µmol) for 1 minute at 25°C. Protein-bound mono-ADP-ribose adducts were isolated by gel electrophoresis (cf. 19).

Legend to Figure 3 (Continued)

B. Labelling of DNA with [^{3}H]-diazo-benzamide was carried out by incubating the same system (enzyme + DNA + histones but no NAD) with 0.6 mM [^{3}H]-diazo-benzamide (5.6 µCi) in an ice-water bath for 30 minutes in a volume of 50 µl.

The reaction was terminated with the gel sample buffer (50 µl) containing 1 mg/ml chloracetyl tyrosine as a diazonium quenching agent and gel electrophoresis performed as above (cf. 19).

LEGEND TO FIGURE 4

Labelling of protein and DNA components of the poly(ADP-ribose) polymerase system present in liver nuclei.

A. Ten µl suspension of liver nuclei (2 mg/ml protein) were allowed to react with [^{3}H]-diazo-benzamide (5.1 µCi, 0.1 mM) at 4°C for 30 minutes and the reaction was quenched as described in Legend of Figure 3, followed by gel electrophoresis (19).

LEGEND TO FIGURE 5

Identification of dGMP as target of 4-diazo-benzamide. Ordinate: pmol [^{3}H]-diazo-benzamide covalently bound per experiment. Abscissa: fraction number.

Experiments 1 and 2. HPLC-separation of spontaneous degradation products of 4-diazo-benzamide ([^{3}H]-labelled), determined under conditions of Experiments 2 to 5. [^{3}H]-4-diazo-benzamide (5 µCi, 0.9 mM) was incubated alone (Experiment 1) or with 10 mM of dAMP, dGMP, dTMP, dUMP (Experiment 2) in 10 mM Tris-HCl pH 7.4,10 mM $MgCl_2$, 0.25 M sucrose medium for 60 min. at 4°C in a total volume of 25 µl. After incubation, 10 µl was injected onto reverse phase HPLC system (Ultrasphere ODS, 5µ, 25 cm x 4.6 mm ID) and developed with a solvent system consisting of buffer A: 0.1 M K phosphate pH 4.25; buffer B: 0.1 M K phosphate pH 4.25 and 50% acetonitrile. Upon sample injection, gradient was started from 100% B in 20 min. using the concave gradient, curve 9. Elution was continued with 100% B for additional 10 min. Flow rate was 1.5 ml/min., fractions were collected every 20 min. The deoxyribonucleotides were located and identified (↓) by their retention time and by their absorption spectra (not shown) recorded by the high-speed spectrophotometric detector (Hewlett Packard 1040-A) for each peak (Experiment 2). The covalent binding of [^{3}H]-4-diazo-benzamide (1 mM, 50 µCi) to DNA present in two ml suspensions of rat liver nuclei (20 mg protein 5.5 mg DNA) was determined following incubation for 60 min. at 4°C in a volume of 2.5 ml of 10 mM Tris-HCl, pH 7.4, 10 mM $MgCl_2$, 1 mM DTT buffer. At the end of incubation, the mixture was diluted two-fold with the buffer, containing the quenching agent chloroacetyl tyrosine (1 mg/ml) and proteins were extracted with 5 ml buffer-saturated phenol. The phenol extraction was repeated and the combined phenol phase re-extracted with water, and the combined water phase extracted twice with 10 ml of diethylether to remove the traces of phenol. Nucleic acids were precipitated with two volumes of ethanol

Legend to Figure 5 (Continued)

in the presence of 0.2 M NaCl overnight at 10°C. The precipitate was collected by centrifugation, dried in vacuum, and traces of RNA hydrolyzed in 3 M NH_4OH at 37°C for six hours. After rotoevaporation, the sample was dissolved into 2 ml of 50 mM Tris-HCl pH 7.4, 20 mM NaCl and the DNA re-precipitated with ethanol as described. Finally the DNA was dissolved in 200 µl Tris-HCl (pH 8.0, 100 mM) containing 100 mM $MgCl_2$ and $CaCl_2$, then completely digested with DNA-se I (1 mg/ml) plus Bal-31 nuclease (25 units per test) for 6 hours at 37°C. The products of digestion were directly injected into the HPLC system and analyzed as described. In Experiment 2 a single deoxynucleotide contained [^{3}H]-diazo-benzamide residue.

In Experiment No. 3, 13 µg coenzymic DNA, dissolved in a final volume of 50 µl buffer (see above) was incubated at 4°C for 60 min. with 0.4 mM [^{3}H]-4-diazo-benzamide (5 µCi), treated as in Experiment 2, (digestion of DNA) and labelled products analyzed by HPLC as described for No. 2.

In Experiment No. 4, 10 mM authentic dGMP (10 mM in 50 µl buffer, pH 7.4) was incubated with 0.9 mM [^{3}H]-4-diazo-benzamide (5 µCi) for 60 min. and the product isolated by HPLC as in No. 4.

In Experiment No. 5, 160 µM labelled dGMP ([^{14}C], 500 µCi/mmol) and 0.9 mM [^{3}H]-4-diazo-benzamide (5 µCi) were incubated exactly as in No. 4, and double-labelled product was isolated by HPLC.

LEGEND TO FIGURE 6

The effects of pre-treatment of coenzymic DNA with 4-diazo-benzamide and 4-diazo-benzoic acid on its catalytic function in a reconstructed system composed of purified poly(ADP-ribose) polymerase protein and coenzymic DNA.

Legend to Figure 6 (Continued)

Conditions of enzyme assays were the same as described in the Legend to Figure 3, except after exposure to the diazo compounds, coenzymic DNA was extensively dialyzed (6 x 4 l H_2O) and reprecipitated by EtOH (4°C) to remove traces of decomposition products prior to enzyme assays. The binding of the [^{3}H]-4-diazo-benzamide was determined by acid precipitation and scintillation spectrometry. Figure 6 (left ordinate) shows rates of ADP-ribosylation in the presence of coenzymic DNA that had been either not treated (top curve), treated with 4-diazo-benzoic acid (1 mM, second curve), or with the same concentration of 4-diazo-benzamide (3rd concave curve). Abscissa = time of pre-treatment of coenzymic DNA. The fourth convex curve shows the covalent binding of 4-diazo-benzamide to coenzymic DNA as indicated by the right ordinate. The specific activity of [^{3}H]-4-diazo-benzamide was 12.5 Ci/mmol. The molarity of the polymerase was 2 nM and of [^{32}P]-labelled NAD 100 nM. (See Legend to Figure 3).

—o——o— enzymatic activity with coenzymic DNA.

—▲——▲— enzymatic activity with coenzymic DNA that was treated with 4-diazo-benzoic acid.

—●——●— enzymatic activity with coenzymic DNA that was treated with 4-diazo-benzamide.

—Δ——Δ— binding of 4-diazo-benzamide to coenzymic DNA.

Fig 1.

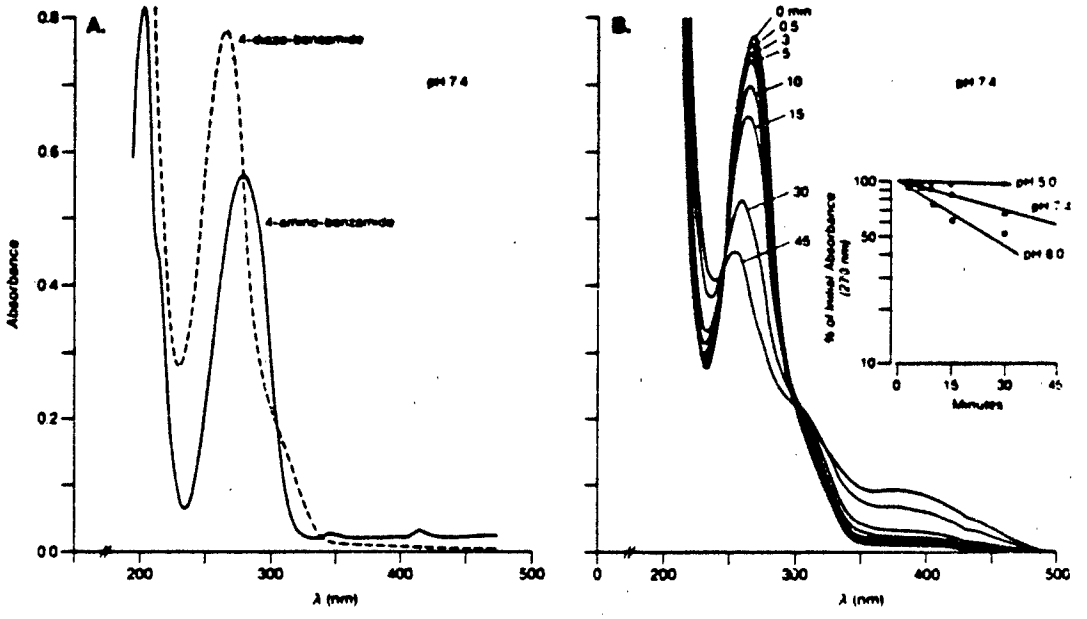
A.
4-diazo-benzamide
4-amino-benzamide
pH 7.4
Absorbance
λ (nm)
B.
0 min
0.5
3
5
10
15
30
45
pH 7.4
pH 5.0
pH 8.0
% of Initial Absorbance (273 nm)
Minutes
λ (nm)

Fig 2

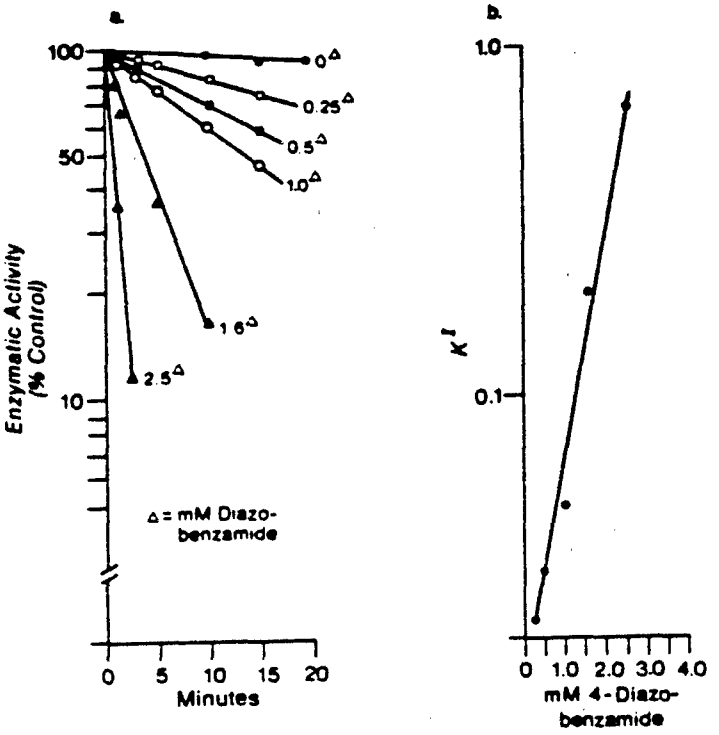
a.
100
50
10
0
0.25
0.5
1.0
1.6
2.5
Enzymatic Activity (% Control)
Δ = mM Diazo-benzamide
0 5 10 15 20
Minutes
b.
1.0
0.1
K^I
0 1.0 2.0 3.0 4.0
mM 4-Diazo-benzamide

Fig 3

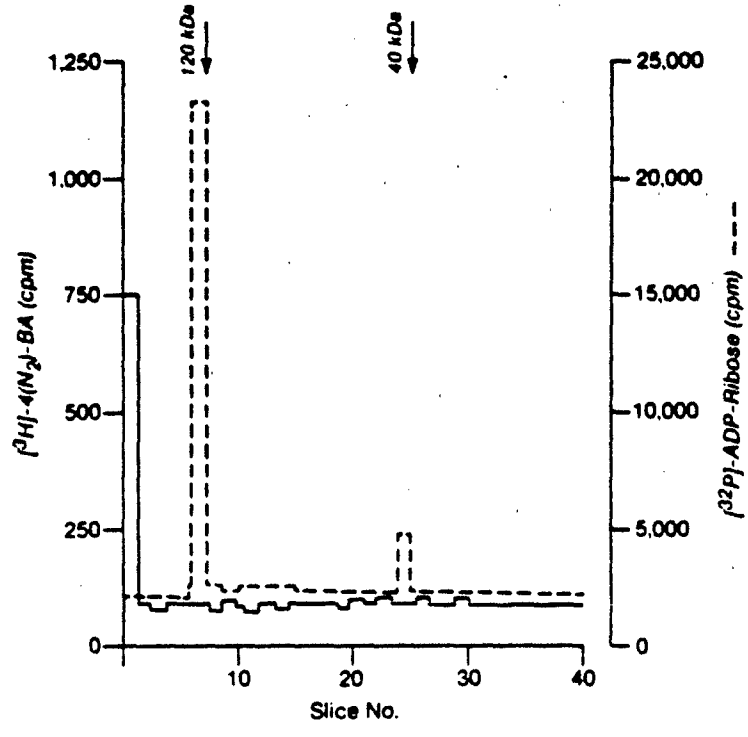
120 kDa
40 kDa
1,250
1,000
750
500
250
0
25,000
20,000
15,000
10,000
5,000
0
10
20
30
40
Slice No.
[3H]-4(N3)-BA (cpm)
[32P]-ADP-Ribose (cpm)

Fig 4.

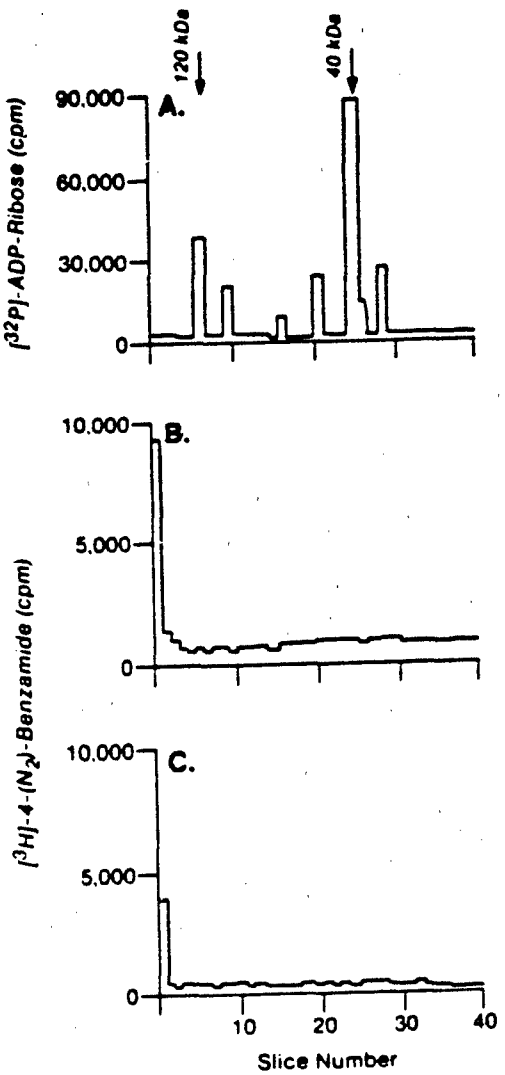
120 kDa
40 kDa
A.
90.000
60.000
30.000
0
[32P]-ADP-Ribose (cpm)
B.
10.000
5.000
0
C.
10.000
5.000
0
[3H]-4-(N2)-Benzamide (cpm)
10
20
30
40
Slice Number

Fig 5.

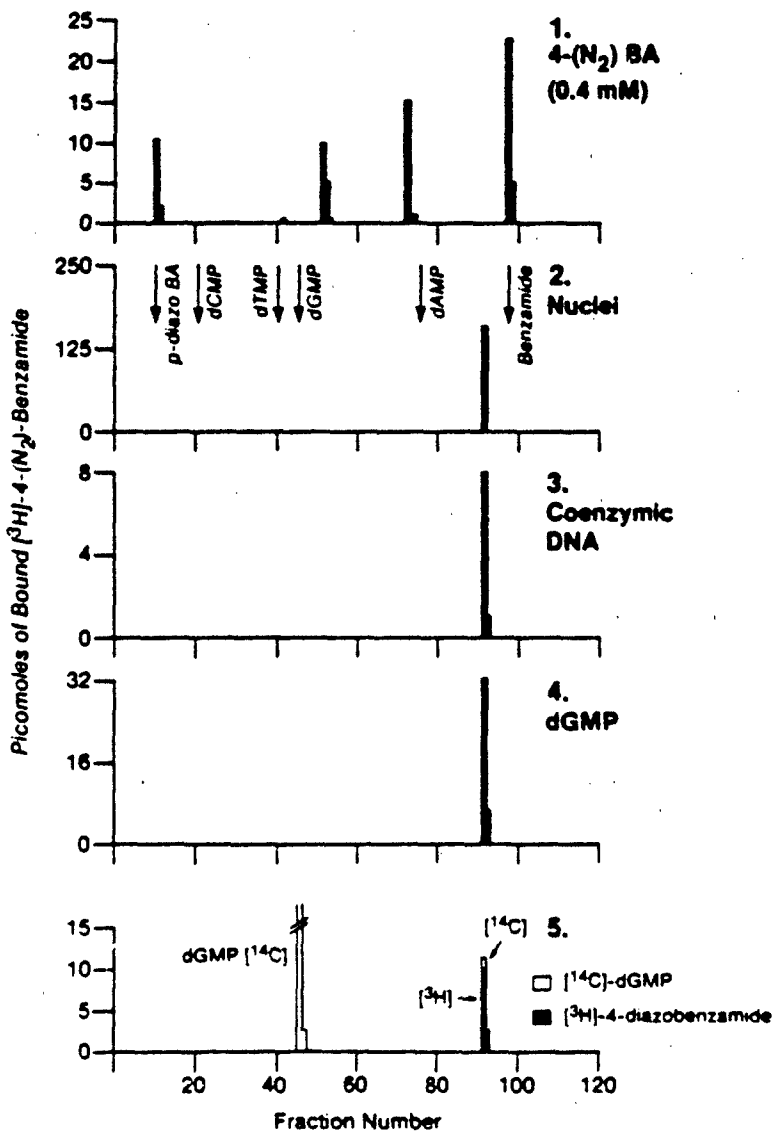
1.
4-(N2) BA
(0.4 mM)
2.
Nuclei
3.
Coenzymic
DNA
4.
dGMP
5.
p-diazo BA
dCMP
dTMP
dGMP
dAMP
Benzamide
dGMP [14C]
[14C]
[3H]
[14C]-dGMP
[3H]-4-diazobenzamide
Picomoles of Bound [3H]-4-(N2)-Benzamide
Fraction Number

Fig 6.

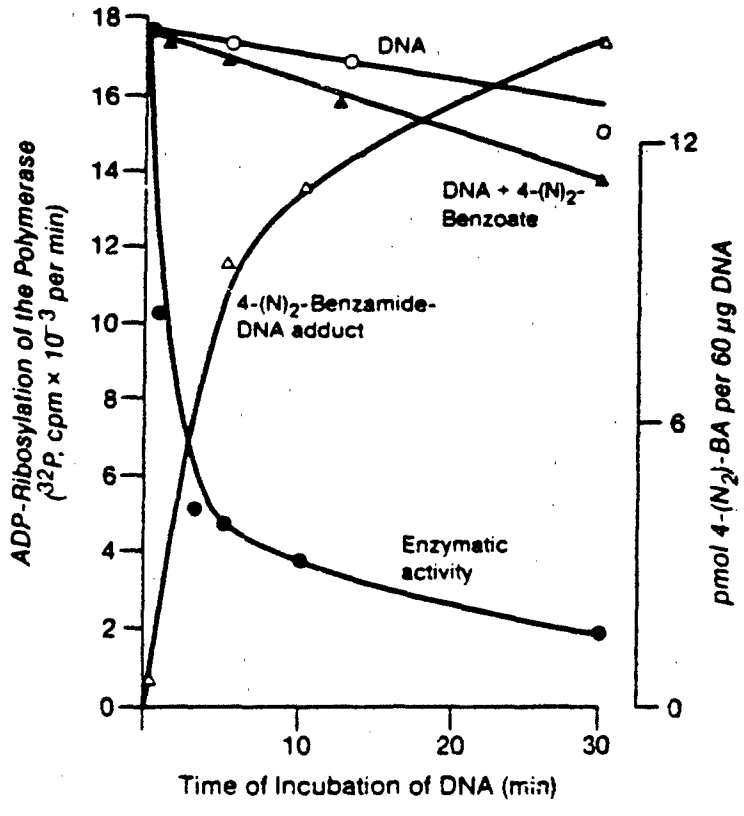
DNA
DNA + 4-(N)2-Benzoate
4-(N)2-Benzamide-DNA adduct
Enzymatic activity
ADP-Ribosylation of the Polymerase (32P, cpm × 10-3 per min)
pmol 4-(N2)-BA per 60 µg DNA
Time of Incubation of DNA (min)
0
2
4
6
8
10
12
14
16
18
0
6
12
10
20
30

TABLE I

Protective effect of benzamide against inactivation of poly(ADP-ribose) polymerase by 1.6 mM 4-diazo-benzamide

	$NaNO_2$ Reagent 1.6 mM	4-diazo-benzoic acid 1.6 mM	4-diazo-benzamide 1.6 mM	Benzamide Concentration mM	ADP-ribose products	
					cpm	n mol ADP-ribose/mg DNA in 1 min.
	-	-	-	-	32,000 (± 18%)	1.60*
	⊕	-	-	-	31,000 (± 16%)	1.55
	-	-	-	7.7	31,000 (± 20%)	1.59
	⊕	-	⊕	-	9,400 (± 10%)	0.46
	⊕	-	⊕	2.30	16,000 (± 12%)	0.79
	⊕	-	⊕	7.7	25,300 (± 20%)	1.27
	⊕	⊕	-	-	27,000 (± 18%)	1.30

*SD is the same as given the preceeding column.

No 4.

PROTEOLYTIC DEGRADATION OF POLY(ADP-RIBOSE) POLYMERASE TO IMMUNOREACTIVE BUT ENZYMATICALLY INACTIVE POLYPEPTIDES

EXPERIMENTAL PROCEDURES

Isolation of poly(ADP-ribose) polymerase: The enzyme was isolated from frozen calf thymus (Pel-Freeze) by the method of Yoshihara, et al. (1978). In short, this protocol involves homogenization of the tissue in 50 mM Tris HCl, pH 7.4, 10 mM EDTA, 1 mM NaN_3, 1 mM glutathione, 0.5 mM dithiothreitol, 50 mM $NaHSO_3$, 0.2 M NaCl, and 0.2 mM PMSF and two sequential ammonium sulfate fractionations of 40% and 80%. After re-suspension of the material from the 80% ammonium sulfate precipitation, the ammonium sulfate was removed by passage of the solution through a Sephadex G-25 column. Further purification steps used elution from DNA cellulose and hydroxylapatite columns. The final step was separation on a Sephadex G-200 column. The "fraction 2" referred to in Figures 3 and 4 was the material obtained from the Sephadex G-25 column.

Antibody production: Antiserum against the purified enzyme was prepared by Antibodies, Inc. (Davis, California) according to the procedure of Silver and Elgin (1976). Purified poly(ADP-ribose) polymerase was obtained from preparative SDS polyacrylamide gels (Laemmli, 1970). The gel band containing the protein was excised, dried and ground to a fine powder. The powder was re-suspended in one volume of phosphate buffered saline (0.01 M sodium phosphate, pH 7.6, 0.15 M NaCl) and emulsified with one volume of adjuvant (Freund's complete adjuvant for the first injection and Freund's incomplete adjuvant for the remaining injections). Rabbits were injected subcutaneously with approximately 100 µg of protein per injection. Injections were made every four weeks for a total of four injections. For all experiments, total serum was used.

Immunoblots: Immunoblots were performed essentially according to Towbin, et al. (1979). Proteins were separated on SDS polyacrylamide gels (Laemmli,

1970) and transferred electrophoretically (20 v 16 hrs) to nitrocellulose (Bio-Rad). All remaining protein binding sites were blocked by incubation of the filter in 0.35 M NaCl, 10 mM Tris-HCl, pH 7.4, 1 mM EDTA, 0.2 mM PMSF (NET buffer) with 10 mg/ml bovine serum albumin for three hours at room temperature. The filter was incubated for six hours at room temperature with the anti-poly(ADP-ribose) polymerase serum diluted 1:25 with NET buffer with 10 mg/ml BSA. Following six washes with NET buffer, the filter was incubated for six hours at room temperature with affinity purified goat anti-rabbit IgG, light and heavy chain specific antibody which had been labelled with [^{125}I] by the use of Iodo-Gen (Pierce). After further washes with NET buffer, the antigen band was visualized by autoradiography.

In vitro poly(ADP-ribosyl)ation of the enzyme and its proteolytic degradation products: the standard reaction mixture contained 150 mM Tris-HCl, pH 8.0, 10 mM $MgCl_2$, 2 mM DTT, 0.2 mM PMSF, 10 µg DNA, 0.1 µg-0.5 µg of protein, and 1.5 mM [^{32}P]-NAD (SA = 600 Ci/mmol) in a total reaction volume of 100-200 µl. The reaction time was 10 seconds. The reaction was stopped by centrifugation of the mixture through a column of Sephadex G-25. The DNA used in the reaction was the DNA which was separated from the enzyme during the purification procedure at the hydroxylapatite column step. This DNA is referred to as coenzymic DNA and is about 50 times more effective as a cofactor than crude thymus DNA. Modified proteins were examined through the use of polyacrylamide gels at pH 5.0 (Jackowski and Kun, 1983) and autoradiography.

RESULTS

Poly(ADP-ribose) polymerase has been isolated from calf thymus by the method of Yoshihara, et al. (1978). Table I presents the results of assays for

the enzymatic activity and protein content of each step in the purification scheme. The protein peak eluted from the hydroxylapatite column which contained the enzymatic activity (Fraction 4). In addition to the 120 kDa band characteristic of the pure enzyme, a small number of other protein bands were apparent. These small impurities can be removed by the passage of Fraction 4 through a Sephadex G-200 column. Analysis by SDS polyacrylamide gel electrophoresis shows a singl protein band of approximately 120 kDa, the pure enzyme (Fraction 5, not shown in Table I). Fraction 4 represents approximately a 300-fold purification of the enzyme from the crude extract. It should be noted that Fraction 2, which will be important to later experiments, is the 40-80% ammonium sulfate precipitate and in a similar electrophoretic analysis this fraction was found to contain many (60-70) protein bands.

Using the 120 kDa protein band as purified on preparative electrophoretic gels, an antiserum was prepared in rabbits against the enzyme (Silver and Elgin, 1976). The specificity of the antiserum for poly(ADP-ribose) polymerase was determined by two methods. In immunoblot experiments, the antiserum bound selectively to the 120 kDa band (Figure 1). In <u>in vitro</u> labelling experiments with the pure poly(ADP-ribose) polymerase, incubation of the enzyme with the antiserum for 5 minutes before addition of [^{32}P]-labelled NAD eliminated any incorporation of the labelled material. The antiserum at a dilution of 1:10 was sufficient to totally eliminate any enzymatic activity while a dilution of 1:100 was only partially effective (Table II).

The use of the antiserum to identify the enzyme on Western blots yielded unexpected findings. Incubation of Fraction 4 of the enzyme preparation for ever relatively short periods of time (minutes) caused a sharp loss of enzymatic activ

(Table III). Examination of the products of such an incubation by SDS gel electrophoresis and Coomassie blue staining revealed the loss of the 120 kDa band in those preparations which had been subjected to incubation. When these protein bands were transferred electrophoretically to nitrocellulose and incubated sequentially with the antiserum against the enzyme and then with [^{125}I]-labelled affinity purified goat anti-rabbit IgG, the 120 kDa band was absent and a new band appeared at approximately 59 kDa (Figure 2b). The new band must represent a relatively stable fragment of the original enzyme band. It is probable that one of the contaminating bands which is co-purified with the enzyme through Fraction 4 (the hydroxylapatite column) is a protease which can degrade the enzyme. To test this hypothesis, the incubation at 37°C was repeated with and without the addition of the protease inhibitor PMSF. SDS gel electrophoretic analysis of the products of this reaction (Figure 2) show that the 120 kDa band seen in Commassie blue staining is retained in the sample with PMSF but lost in the sample without the protease inhibitor (compare Figure 2a and 2b). Enzymatic analyses of these two preparations confirm that the active enzyme is present only in the samples with PMSF.

Because the enzyme has the unique property of being able to modify itself by auto-poly-ADP-ribosylation, it was of interest to determine if the 59 kDa fragment contained modification sites. To accomplish this, the enzyme was auto-modified and then allowed to degrade as before. Analysis of the products of this reaction on SDS gels and autoradiography revealed that the 59 kDa band was ribosylated (Figure 3). Furthermore, this same experiment revealed the stability and rate of formation of the 59 kDa fragment. Within a period of 15 minutes, the 59 kDa fragment appeared and was not further degraded during further incubation for four hours.

Fraction 2 (Table I) was used as a source of the protease. When aliquots of Fraction 2 were added to Fraction 4 or Fraction 5 enzyme, a series of breakdown products including the 59 kDa band were seen. Comparison of the in vitro ribosylated breakdown products with the peptides that give a cross-reaction with the antiserum made against the pure enzyme show good correspondence at the higher molecular weights (Figure 4a) ADP-ribosylated proteins, (Figure 4b) Western immunoblot.

SUMMARY

We have isolated poly(ADP-ribose) polymerase from calf thymus and examined some of the molecular and immunological properties of the enzyme. An antiserum prepared against the electrophoretically purified enzyme was prepared in rabbits and shown to be specific for the enzyme by the use of Western immunoblots and by inhibition of the enzyme in in vitro reactions. The antiserum was then used to identify the enzyme and its proteolytic breakdown products in various experiments. It was found that the incubation of the relatively pure (Fraction 4) enzyme result in a rapid loss of enzymatic activity along with a loss of the 120 kDa band characteristic of the pure enzyme. By the use of the antiserum and Western blots we were able to show the appearance of a proteolytic product as a band at 59 kDa. The specificity of the antiserum for the enzyme identifies the 59 kDa protein band as a breakdown product of the enzyme. Further experiments showed the 59 kDa fragment to contain sites which are poly ADP-ribosylated and to be remarkably stable to further degradation. That the degradation and loss of enzymatic activity were preventable by the use of PMSF indicates that there is a protease that co-purifies with the enzyme through the hydroxylapatite column steps. The nature of the columns in the purification protocol is such that primarily DNA binding proteins are retained (DNA cellulose, hydroxylapatite). It appears that

the protease in this case must be able to bind to these types of columns. The addition of Fraction 2 to Fraction 4 enzyme preparations revealed a series of breakdown products including the 59 kDa fragment. It is likely that the same protease is present in the Fraction 2 mixture but in a more impure stage and therefore the presence of a number of other breakdown products indicates an incomplete reaction. The relative stability of the 59 kDa fragment may indicate that it contains ADP-ribose acceptor and epitope domains of the enzyme.

LEGENDS

Figure 1

Identification of the enzyme and its primary breakdown product by the immunoblot technique. Samples of the pure enzyme (Fraction 5) and its breakdown products were separated on a 7.5% polyacrylamide SDS gel and transferred electrophoretically to nitrocellulose. The nitrocellulose filter replica was incubated sequentially with rabbit anti-poly(ADP-ribose) polymerase serum and [^{125}I]-labelled goat anti-rabbit IgG.

a. Pure enzyme (Fraction 5) as visualized by Coomassie blue.

b. The breakdown products resulting from incubation of Fraction 4 enzyme without protease inhibitor.

c. Immunoblot of pure enzyme.

d. Immunoblot of the post-incubation sample showing the single cross-reacting band at 59 kDa.

Figure 2

Degradation of the enzyme is preventable by the addition of the protease inhibitor PMSF.

Samples of the enzyme (Fraction 4) were incubated one hour at room temprature with (a) and without (b) PMSF. Examination of the samples on 7.5% polyacrylamide SDS gels reveals the 120 kDa band characteristic of the intact enzyme in those samples with the protease inhibitor and the loss of that band and the appearance of a 59 kDa band when PMSF is omitted.

Figure 3

The 59 kDa primary breakdown product contains sites of poly(ADP-ribosyl)-ation.

Samples of the enzyme (Fraction 4) were allowed to auto-modify themselves with [^{32}P]-NAD briefly and then were exposed to an aliquot of Fraction 2 for: (a) zero time; (b) 15 minutes; (c) 1 hour; (d) 2 hours and (e) 3 hours. The breakdown of the enzyme yields a labelled product at 59 kDa and another at approximately 70(?) kDa.

Figure 4

A number of breakdown products of the enzyme can be identified by immunoblots and auto-labelling of the enzyme.

Enzyme (Fraction 4) was allowed to auto-label briefly as outlined in Experimental Procedures and then incubated with an aliquot of Fraction 2. The breakdown products were separated on a 10% polyacrylamide SDS gel and immuno-blotted. The resulting autoradiograms enable a comparison of the autolabelled fragments (a) with those identified by the specific antiserum on immunoblots (b).

FIGURE

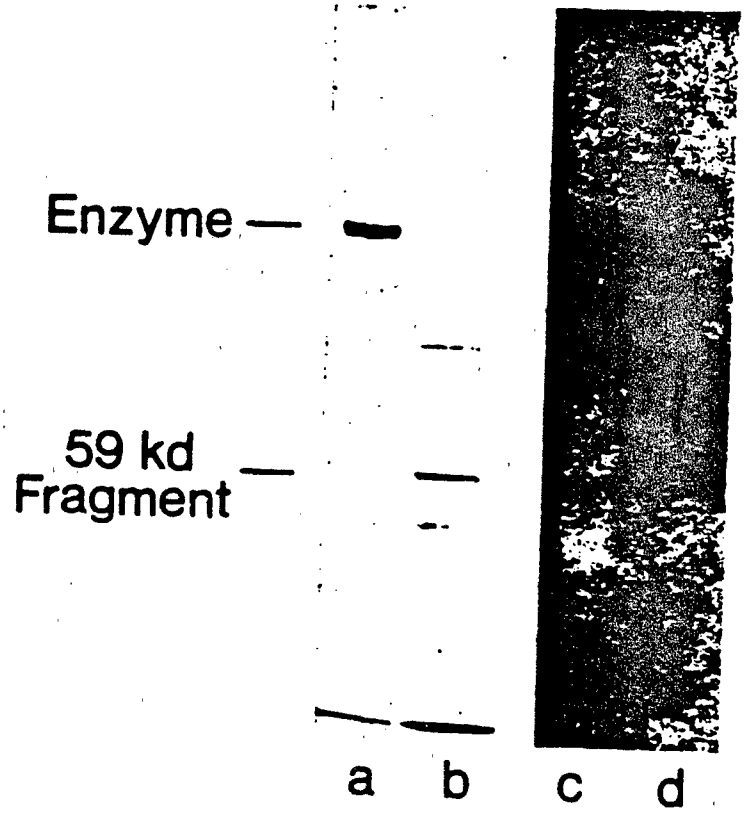

Fig 1.

FIGURE 2.

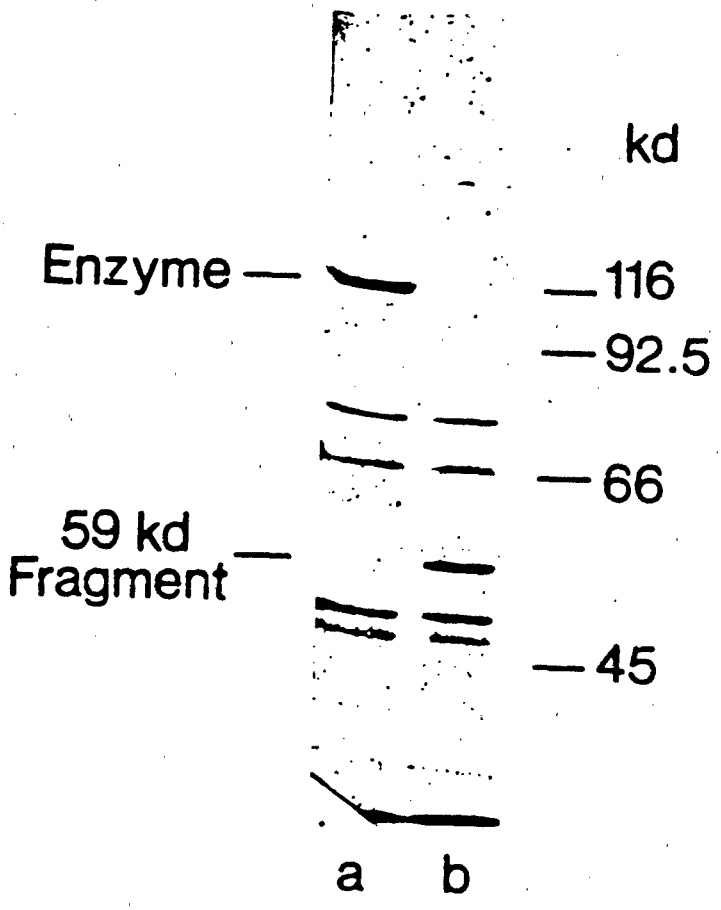

Fig 2.

FIGURE 3

a b c d e

Fig 3

FIGURE 4

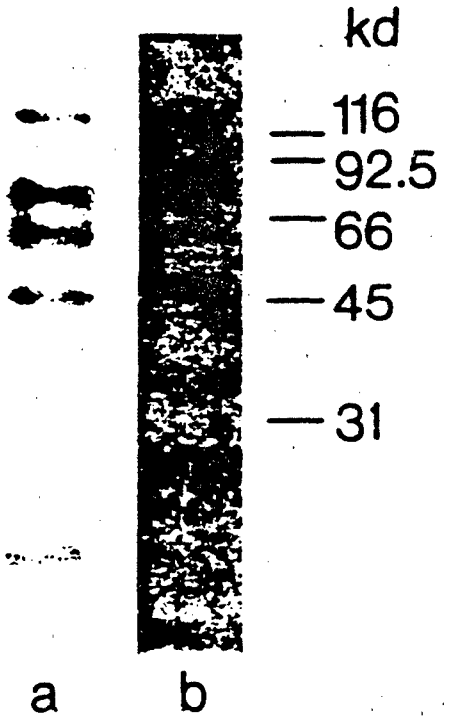

Fig 4

Table I: Purification of poly(ADP-ribose) polymerase.

	Fraction	Protein (g)	Total Activity	Specific Activity
I	crude extract	54	1.2×10^{8}	2.2×10^{6}
II	ammonium sulfate 40-80% fraction	8.6	6.5×10^{7}	7.6×10^{6}
III	DNA cellulose	0.17	5.4×10^{7}	3.2×10^{8}
IV	hydroxylapatite	9.8×10^{-3}	6.3×10^{6}	6.4×10^{8}

TABLE II

Inhibition of poly(ADP-ribose) polymerase by a specific antiserum.

	Recovered As cpm Acid Precipitate
pre-immune serum	21000
1/100 dilution of specific antiserum	4500
1/10 dilution of specific antiserum	0
undiluted antiserum	0

TABLE III

Assay of enzymatic activity of the purified enzyme and its proteolytic degradation products.

	cpm Recovered As Acid Precipitate
pure enzyme (Fraction 4)	24,000
breakdown products	0

References

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NO. 5

Synthesis of deoxyoligonucleotide 8-mers as models of coenzymic DNA and tests for base sequence specificity in the activation of poly(ADP-ribose) polymerase.

To help clarify the mechanism and biological role of poly(ADP-ribose) polymerase we have initiated work in the chemical synthesis of model DNA molecules in order to test the nature of the interaction of the enzyme with such molecules. In our initial study we synthesized DNA duplexes 8-base pairs long, this being the minimal length known to function effectively as coenzymic DNA.

We have chosen to synthesize 8-mers of potential biological significance. Therefore, we chose three kinds of base sequences which could be useful for this purpose. We designate the three synthetic duplexes as A, B and C. Duplex A is a consensus sequence predicted in natural DNA to bind dexamethasone receptor protein (see 6). Duplex B is a modification of A wherein at two sites there is a transversion of T to A residues, in order to test biochemical sensitivity to relatively subtle changes in base sequence. Duplex C is a sequence of alternating purine-pyrimidine bases found in the enhancer region of SV_{40} DNA. It is believed that such a sequence forms Z-DNA (left-handed double helix) whose structure may be related to transcriptional activation. Specifically, the three duplexes synthesized (where one strand is presented) are:

A 5' A - G - A - T - C - A - G - T 3'

B A - G - A - A - C - A - G - A

C G - C - A - T - G - C - A - T

Formation of double-stranded DNA of course first entails synthesis of single strands of the desired base sequence plus strands of the exact complementary sequence. The method of deoxyoligonucleotide synthesis which we employ

is the solid-phase phosphoramidite method currently utilized in commercial automated DNA synthesizers, but which we effectively perform manually on a sintered glass filter. In the construction of a given strand, the series of synthetic reactions begins at the desired 3' terminal group, which is commercially available in the form of a deoxynucleoside (A,G,C or T) covalently bound by way of its ribose 3'-oxygen to controlled-pore glass beads. These beads are reacted while suspended on the sintered glass filter. The 5'-OH of the bound nucleoside is allowed to condense with the phosphorus atom of the next desired group (A,G,C or T) which is in the form of a specifically protected deoxynucleoside - 3'-phosphoramidite. The condensation yields a glass-bound dinucleoside phosphite which, on the filter, is washed free of impurities and excess reagents. The 5'-OH of this species is then deprotected and allowed to condense with the next desired deoxynucleoside - 3'-phosphoramidite to give the glass-bound trinucleoside phosphite, and the reaction and washing cycles are continued until the desired deoxyoligonucleoside phosphite species is attained. Oxidation of the phosphorus atoms from phosphite to the phosphate yields the target deoxyoligonucleotide which is removed from the glass beads by base hydrolysis and freed of auxillary protecting groups. The oligomer is then separated from failure sequences by ion-exchange HPLC, with elution by phosphate buffer, and desalted on a small reversed-phase column to give the target oligomer in high purity. In our procedure, we start with 1 Mmole of glass-bound mononucleoside for each synthesis and obtain an average of 650 µg of pure single-strand DNA 8-mer, which is ample for biochemical experiments.

Duplex DNA is formed by combining equivalent amounts of complementary strands in an annealing buffer (50 mM NaCl, 6.6 mM Tris (pH 7.6), 6.6 mM $MgCl_2$ and 1.0 mM dithiothreitol), heating briefly at 100°C and cooling slowly to room

temperature. The duplexes were labelled at both ends with [^{32}P] using γ-[^{32}P]-ATP and T_4 polynucleotide kinase. The presence of [^{32}P]-duplexes was confirmed by polyacrylamide gel electrophoresis (20%) and subsequent autoradiography. Parallel samples of [^{32}P]-labelled single-strand 8-mers were run as controls.

5(A)

Studies on Interaction of Poly(ADP-Ribose) Polymerase with DNA

INTRODUCTION

Although a large body of information is known about the catalytic properties of the eukaryotic enzyme poly(ADP-ribose) polymerase, very little information is available regarding the enzyme DNA interaction. Our main objective is to elucidate the mechanism(s) of interaction of the enzyme with DNA.

RATIONALE

In order to study the interaction of poly(ADP-ribose) pol erase with DNA in vitro, a sensitive assay for binding was developed. This assay (see below) will allow us to detect binding of the enzyme to DNA in extremely small quantities of enzyme and DNA. This assay will also permit us to (a) carry out quantitative measurements of the interaction and (b) assess the binding efficiencies of various DNA molecules which might serve as effectors of catalytic activity and therefore look at the relationship between catalytic activity and binding effeciency and (c) do competition assays to compare the relative binding efficiencies of various DNA molecules.

Finally, we would like to use this information gathered from binding studies to solve two major questions in the reaction of poly(ADP-ribose) polymerase-DNA interaction. These questions can be paraphrased as followed:

1. It has often been speculated that the enzyme binds to ends and nicked regions in the DNA duplex. We will try to answer this question by doing eronuclease protection experiments (for ends) and DNA polymerase (Klenow fragment) fill-in experiments (for nicks).

2. Another interesting aspect of the enzyme - DNA interaction is the question whether the enzyme recognizes any specific sequences on the DNA molecules. We propose answering this question by "DNase I foot-printing".

EXPERIMENTAL DESIGN

Nitrocellulose filter binding assay. An enzyme-DNA binding reaction was done by mixing appropriate quantities of the purified enzyme with [^{32}P]-labelled DNA in a binding buffer (Oghushi, 1980 JBC) and the reaction mixture is filtered through nitrocellulose (BA85, Schleicher & Schuell) filter discs. The retention of radio-label is quantitated after repeated washing of the filter disc with DMSO containing binding buffer (to remove unbound radioactivity) and counting the radioactivity in a scintillation counter. Normally, in the absence of the enzyme less than 5 - 10% of the total input counts are retained on the nitrocellulose filter under these conditions. Appropriate controls were employed in each case and all experiments were done in duplicate.

Competition assays. To quantitate the strength of binding of the enzyme to DNA, unlabelled DNA from sources like native co-enzymic DNA, phage A DNA cut with Hind III, and PBR 322 DNA but with Hind III was used to compete with labelled DNA, (S_1 nuclease digested co-enzymic DNA, Genomic 14C cell DNA cut with Mbol, λ Hind III fragments, PBR 322 Hind III digest, ϕ X 174 RFI Hae III fragments, synthetic octomer duplexes). In each case, the relative binding efficiency (expressed as the ratio of emp retained when unlabelled DNA is present vs. cpm retained when unlabelled DNA is not present expressed as percent of native co-enzymic DNA (taken as 100%)) was calculated.

PRELIMINARY EXPERIMENTAL RESULTS

1. Demonstration of filter binding. $\gamma[^{32}P]$-DNA bound to NC filters was eluted from filter discs by soaking them in elution buffer and ethanol, precipitation of DNA and agarose gel electrophoresis and autoradiography. Autoradiography showed that when enzyme is present in the fragments of the input DNAs were retained. When the enzyme is absent, there was virtually no $[^{32}P]$-DNA fragments present in the autoradiogram.

2. Estimation of the number of enzyme molecules bound per molecule of $[^{32}P]$-labelled co-enzymic DNA. Two titration experiments were done for this purpose.

(a) Increasing quantities of enzyme were used in filter-binding assays with the S_1 nuclease digested $[^{32}P]$-co-enzymic DNA held constant.

(b) Increasing quantities of S_1 digested $[^{32}P]$-co-enzymic DNA were used against a fixed concentration of the enzyme.

In both cases, K_{diss} was estimated (one-half of saturation constant). Average molecular weight of co-enzymic DNA was estimated as 350 bp (from agarose-gel electrophoresis). Using these numbers it was estimated that on an average, 14 molecules of the enzyme could be bound to one DNA molecule of an average length of 350 bp, assuming that all enzyme molecules in a given reaction mixture are active. This number closely compared with other estimates (Hashida, 1979) for native co-enzymic DNA obtained by other methods which relate to the catalytic activity. We assume that since the titration curves were sigmoidal in nature, (a) the DNA binding form of the enzyme is a multimer; (b) the enzyme binds cooperatively.

3. Competition assays. When various DNA's differing in their sequences ends and lengths are used as unlabelled competitors against co-enzyme, genomic,

phage, plasmid DNAs and synthetic octanucleotides we found that the binding efficiencies differed. For example, native co-enzymic DNA bound strongly to the enzyme followed by MboI digest of 14C genomic DNA and synthetic duplexes: (A,B,C) (see section 5 above).

On the other hand, phage and plasmid DNA did show weaker binding by the enzyme. This difference could be a reflection of the availability of specific sequence sites and the types of ends both of which have been implicated in determining binding affinities of the enzyme to DNA. One important fact to emerge from these experiments is that in general there is a close correlation between binding efficiencies and catalytic activity although there could be some exceptions.

NO. 6.

Regulation of Dexamethasone Induced Ras-gene Expression by Coumarin in 14-C Rat Fibroblasts.

The most direct evidence in favor of a real biological function of poly ADP-ribosylation emerged from two experimental fields, developed in our laboratory (see Progress Report, 1984). The first: participation of poly ADP-ribosylation in hormone mechanisms; the second: the prevention of neoplastic transformation by drugs that react with the poly(ADP-ribose) system. Since neither of these results - albeit highly persuasive - contained true molecular mechanisms, it was mandatory to develop an experimental model, that in macro-molecular terms can provide a more detailed insight. The 14-C cell, containing a specific gene-regulatory construct, provided an experimental tool for the testing of both experimental results (see above).

BACKGROUND

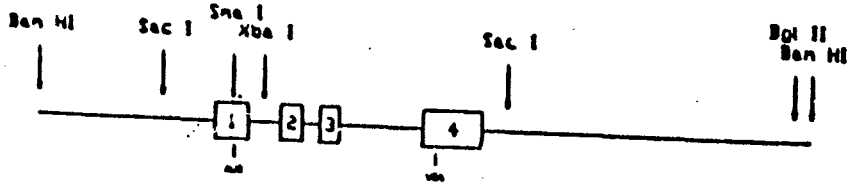
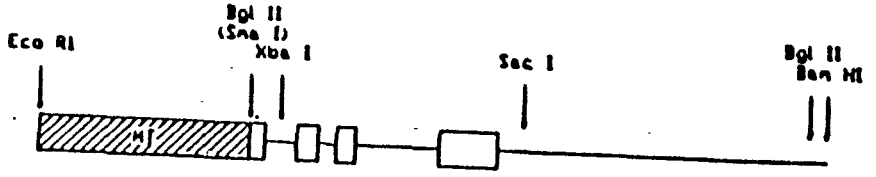
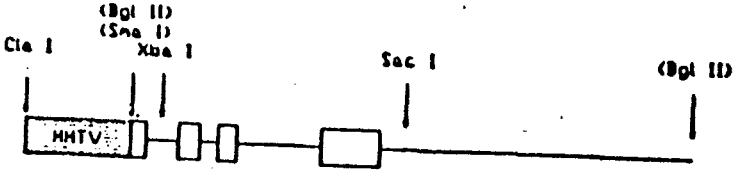
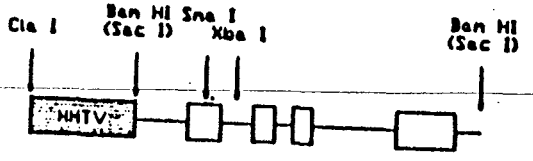
It has beem well-established that glucocorticoids (dexamethasone, abbreviated Dex.), stimulate DNA transcription by the specific binding of DEX-receptor complex to a specific DNA-sequence (see "consensus" octadecxy nucleotide synthesis in 5), which is part of a functional "hormone responsive element" in DNA. If mammary tumor virus DNA sequence (MMTV), which is a retrovirus sequence, is introduced downstream from the hormone receptor DNA element, DEX will stimulate the MMTV-promoter (increase MMTV-mRNA) within minutes, i.e., a specific gene activation sequence is established. Combining this DNA construct with EJ-ras oncogene and a restriction fragment promoter into one DNA sequence is illustrated in the Figure yielding the pMMTV-EJ-ras-pV1C3-neo construct that has been transfected in a rat fibroblast to yield 14C cell line (by William Lee,

Department of Microbiology, UCSF). Sarc-gene can be also introduced, substituting EJ-ras. The abbreviations shown in the Figure are denoting the names of restriction endonucleases used in the gene construct synthesis. This scheme is shown in the Figure on the next page. The specific property of 14-C cell line is that it behaves like a normal fibroblast in cell culture, except addition of 10^{-7}M Dex in two days converts it into a malignant phenotype. This phenotype conversion is readily seen by soft-agar colonies (see Progress Report, 1984), just like in chemical or radiation-induced carcinogenesis and mechanisms of neoplastic transformation are readily studied in this cell line.

In confirmation of earlier results (see Progress Report, 1984) Dex decreases apparent enzyme activity as tested in vitro and augmented polymer content (determined by the new method, see 1, this report). This paradox is explained by (a) Dex-induced conformation change in chromatin, making ADP-ribose sites inaccessible to external NAD (as by necessity done in enzyme assays) but increasing in vivo ADP-ribosylation from intranuclear NAD. Results are summarized below.

RESULTS

Cell lines containing an inducible EJ-ras oncogene have been developed in order to study the effect of drugs which interact with the poly(ADP-ribose) polymerase system on transformation. These drugs are isoquinoline (ISOQ) and coumarin (COUM). The 14-C cell line was derived by transfection of R-1 cells with a plasmid containing a 4.8 kb Sma-Bgl II segment of EJ-ras linked to a mouse mammary tumor virus promoter. 14-C cells display morphologic transformation and anchorage-independent growth after a 48 hr exposure to 0.1 μM dexamethasone (DEX). Tumor incidence and size can be reduced when (1) cells are exposed for 72 hours to 1 μM ISOQ or 100 μM COUM ± induction with DEX, prior to

Plasmid Designation	Construction	Selected Rat-1 Transfected Cell Lines
EJras		
pMT-EJras (Sma-Bam) in pBR 322		15-A
pMMTV-EJras (SMa-Bgl) in pCV103 neo		14C
pMMTV-EJras(Sac) in pCV103 neo		6B1

injection and when (2) rats are fed with 5 μM ISOQ or 200 μM COUM in the water supply ad libitum for 1 week prior to injection. Concentrations of ISOQ and COUM were non-toxic to cells or rats. In rats injected with cells not exposed to DEX, ISOQ reduced mean tumor weight (wt) by 90% vs. control ($p < 0.01$), and COUM produced an 81% reduction ($p < 0.01$). To investigate the mechanism by which these drugs inhibit tumorigenesis, EJ-ras mRNA levels were examined. These drugs had no effect on mRNA levels. Poly(ADP-ribose) content of 14-C cells was 0.176 μmol ± 10%/10^6 cells before DEX treatment and 0.274 μmol/10^6 cells after DEX treatment. An increase in polymer content and decrease in polymerase enzyme activity appears to correlate with transformation.

SUMMARY

(a) DEX-transformation of 14-C cells coincides with characteristic alberation in poly ADP-ribosylation, supporting the view that the poly(ADP-ribose) system is instrumental in the as yet unknown process of regulation of gene activation within a defined DNA sequence (as it exists in 14-C cells).

(b) DEX-induced transformation and *in vivo* tumoregenesis are prevented by the antitransforming drugs: coumarin, previously shown to prevent chemical carcinogenous (see Progress Report, 1984).

These results are the basis of continued research aimed at genetic-chemical mechanisms.

NO. 7

Mathematical Model For Poly ADP-Ribosylation

Before presenting for the poly ADP-ribosylation of an initiator protein, a formal kinetic scheme and its possible elaboration, we survey in qualitative terms some relevant features of the kinetics. These are taken up in more detail elsehwere in this report. Some of these features are shared with uncatalyzed polymerizations as well as with macromolecular syntheses brought about by other enzyme systems.

As with most other polymers formed by enzyme catalysis, poly(ADP-ribose) synthesis entails growth exclusively by accretion of monomers to a single chain (nucleic acid ligase reactions where two macromolecular substrates are linked in the catalytic process provide exceptions to this generality). However, there is with poly ADP-ribosylation a distinct initiation reaction where a bond of a different type than that manifest in the polymer is established as a protein initiator site. As described elsewhere this primary step occurs reversibly at the nanomolar level of NAD while the subsequent elongation carries it along to macromolecular product at the much higher micromolecular concentrations of monomer where the polymerization is conveniently studied. This behavior encourages a formulation where an initiator ADP complex may be treated as a "simple" precursor of macromolecular synthesis with the details of kinetics of initiation treated seaprately. The fact that no direct reaction between poly (ADP-ribose) chains occurs leads to the natural supposition that beyond a certain length the kinetic parameters, i.e., maximum velocities and Michael's constants and the rate constants of which they are compounded should be sensibly independent of polymer size. An account of a highly simplified kinetic treatment

embodying these propositions appears on subsequent pages.

With a macromolecular species serving as the initiator for the polymerization, a heterogeneity of initiation sites might not be unexpected. This can mean that the "clear cut" temporal division into initiation and elongation can be complicated by a slow initiation of chains at some sites taking place while other sites are well into the elongation phase. Evidence for such a complication is probably already at hand.

One aspect of enzyme catalyzed polymerizations probably without a counterpart in uncatalyzed polymer synthesis is provided by the so-called processive attack of an enzyme on a macromolecular substrate. In such a situation there is a probability of an enzyme molecule once having formed a complex with a given polymer chain remains in intimate association with it while the elongation (or degradation) of the chain proceeds. After a finite number of steps (presumably statistically distributed), the complex breaks down. This processive mode of reaction has been invoked frequently for the enzymes of nucleic acid metabolism.

The norm for the chain size distribution in macromolecular synthesis of the type exemplified by poly ADP-ribosylation is the Poisson distribution. Heterogeneity of initiation sites will lead to departures from this characteristic form. Provision for a processive reaction scheme can yield a Poisson distribution but with the basic parameter modified by inclusion of the reassociation probability.

KINETIC ANALYSIS OF POLY ADP-RIBOSYLATION

In a previous report (AFOSR program report 1985) a kinetic scheme for the enzyme catalyzed synthesis of poly(ADP-ribose) from NAD was advanced which involved an ordered sequence of five steps with ten forward and reverse rate constants taken to be independent of chain length. The assumption of a steady rate in the various enzyme substrate complexes led to a set of differential equations of the following form:

$$-\frac{d\{I(ADP\text{-}R)_n\}}{dt} = \bar{\nu}_f\{I(ADP\text{-}R)_{n-1}\} - (\bar{\nu}_f + \bar{\nu}_b)\{I(ADP\text{-}R)_n\} + \bar{\nu}_b\{I(ADP\text{-}R)_{n+1}\} \quad (1)$$

In equation (1)I refers to the initiator of the polymerization and I(ADP-ribose)$_n$ etc. are the concentrations of species of variable chain length n. The "kinetic constants" v_b are composites of the (1) maximum velocities (2) the Michaelis constants and (3) the concentrations of NAD and the product nicotinamide, NA.

As is customary, the enzyme kinetic parameters (1) and (2) are in turn readily expressible in terms of the ten rate constants of the proposed reaction mechanism (Bloomfield, et al., 1962; King and Altman, 1956).

Two additional differential equations must be specified to complete the system. The rate of disappearance of monomer (NAD) and appearance of by-product (NA) are given by:

$$-\frac{d\{NAD\}}{dt} = \frac{d\{NA\}}{dt} = \nu_f \sum_{n=0}^{\infty} \{I(ADP\text{-}R)_n\} - \nu_b \sum_{n=1}^{\infty} \{I(ADP\text{-}R)_n\}$$

$$= \nu_f \{I\}_o - \nu_b \sum_{n=1}^{\infty} \{I(ADP\text{-}R)_n\} \qquad (2)$$

The differential equation for the initiator concentration also has a special form, viz.

$$\frac{d\{I\}}{dt} = -\nu_f \{I\} + \nu_b \{I(ADP\text{-}R)\} \qquad (3)$$

The same "kinetic constants" ν_b and ν_b appear in (2) and (3) as in the set of equations (1). This restriction particularly as it applies to equation (2) and (3) will be discussed further below.

Substantially, the same system of equations had been proffered earlier to describe enzyme catalyzed single strand polynuclotide synthesis from nucleoside triphosphates initiated by a primer (Peller, 1977). Both the solution ignoring the back reaction and the approach to equilibrium were discussed therein. The former yields a Poisson distribution of sizes (Flory, 1940, 1953) while the latter gives rise ultimately to a most probable distribution of species (Miyake and Stockmayer, 1965).

For the enzyme catalyzed synthesis of poly(ADP-ribose) it was deemed likely that the reverse reaction could be ignored. The system of differential equations could then be integrated to yield for the weight fraction of n-mer

$$W_n = \left[\nu^{n-1}/(n-1)! \right] (exp - \nu) \qquad (4)$$

The parameter is both the number average degree at polymerization $\langle n \rangle$ and approximately the value at the chain length at which W_n is a maximum. It can be expressed as:

$$\nu = \langle n \rangle = \int_0^t r_f \, dt \qquad (5)$$

All the particularities of the system reside in r_f and hence in ν.

Referring all the species to that of maximum likelihood ($W_{n_{max}} \approx 1/\sqrt{2\pi\nu}$) on utilizing the Stirling's approximation for the factorial in eq'n (4)), yields

$$W_n / W_{n_{max}} \approx (\nu/n)^{n+1/2} exp(n-\nu) \qquad (6)$$

Moderate agreement with the experimental data was obtained with this expression and ν =70 around the maximum (Kun, et al., 1983) but marked departures occurred at shorter chain lengths.

In order to facilitate further discussion of this problem, we note that in the Gaussian limit the Poisson distribution takes on a form which can be expressed as

$$W_n / W_{n_{max}} \approx exp - \nu/2 \left[(n/\nu) - 1 \right]^2 \qquad (7)$$

(Margenau, and Murphy, 1948). One other representation, a gamma distribution function, employed to describe linear polymers by Schulz and Zimm gives for the weight fraction

$$W_n = \left(y^{z+1}/z!\right)\left(n^z\right)\left(\exp -yn\right) \qquad (8)$$

Here $\nu = n_{max} = z/y$. For $z = 1$, this expression yields the broad most probable distribution which awaits any polymerization at equilibrium. By the arguments utilized above this distribution function provides for the ratio of weight fraction of species n to that at the maximum the form

$$W_n / W_{n_{max}} \simeq \exp -z/2\left[(n/\nu) - 1\right]^2 \qquad (9)$$

where ν now plays the role of the second parameter. When z becomes large this distribution clearly narrows, and at $z=\nu$ it is, of course, indistinguishable from the Gaussian limit of the Poisson distribution. (eq'n (7)).

The symmetry about $n=\nu$ in eq'n (9) is a consequence of the Gaussian limit as in the more familiar eq'n (7).

The broadened distribution of chain lengths attributed to a heterogeneity of initiation sites and alluded to earlier (Kun, et al., 1983) might in principle be fitted by a superposition of a number of Poisson distributions with different values of ν. Widening due to the onset of the reverse reaction normally dealt with by the inclusion of the term in V_b might find formal representation by eq'ns (8) and (9) with both ν and z time dependent. In the latter instance, ν would of course increase with time while z would decrease from ν and move toward unity.

Two remaining possible causes of breadth in the distribution must be discussed. The first is a difference in the kinetic parameters for the initiation step from those of the further elongation steps. This can occur for polymerizations not catalyzed by enzymes. However, for the latter case, the effects may be more striking and may extend to the steps involved in the synthesis of early oligomers. The second cause arises from the phenomenon of processivity in catalytic reactions and is expected to be most marked in heterogeneous or surface catalysis or with enzymes. Processive reaction is here meant to include reactions in which the catalyst has a finite possibility of remaining associated with the macromolecular substrate after addition of a monomer unit and is thus in a "good" position to catalyze the acquisition of an additional monomer.

Taking up the first stated cause, we can argue inasmuch as the initiation reaction must involve a functional group of an amino acid side chain in the acceptor protein it is perforce chemically distinct from the subsequent elongation steps in the formation of the poly(ADP-ribose) chain. Hence, an initiation reaction might be expected to be governed by different rate constants than those applying to the lengthening of the chain. This has been observed experimentally (Bauer, Hakam, Kun, manuscript in progress, 1985). Ignoring the reverse reaction, i.e., ($v_b = 0$), we must modify the first equation of the set (1) and equations (2) and (3) by replacing v_f by v_f^i which is distinct from v_f^p or the propagation "rate constant" which appears for all those reactions involving dimers and larger species.

Some studies (Gold, 1952, 1959) directed toward extending Flory's original arguments (Flory, 1940, 1953) can serve as a partial guide here. No simple form for the distribution at all times seems to emerge. For

$r \equiv (v_f^p / v_f^i) >> 1$

- in our development an initiation much slower than the subsequent elongation rates, we can conclude from these investigations that the polydispersity index $\langle n^2 \rangle / \langle n \rangle^2$ only seems to attain a maximum of about 1.3 to 1.4 for r varying between 1 and 10^6. The quantity $\langle n^2 \rangle / \langle n \rangle^2$, the ratio of the weight, $\langle n^2 \rangle / \langle n \rangle$, to number average degree of polymerization, $\langle n \rangle$, for a Poisson distribution approaches unity. This in some ways surprisingly small effect of r on the polydispersity warrants reinvestigation particularly if v_b^i is not negligible but v_b^p remains so. Examining the question of reversibility of the initiation step would require machine computation more elaborate than that performed in the studies cited here. Additional numerical complications must be faced if variation of the kinetic parameters with chain length for the synthesis of the early oligomers is contemplated.

We turn now to the question of the role of processive reaction on the kinetics and size distribution of the poly(ADP-ribose) species. It should be noted that the first study and treatment of this type of reaction dealt with the Beta-amylase catalyzed degradation of amylose chains (French and Bailey, 1957). Subsequently, this mode of reaction has been held to obtain for polynucleotide phosphorylase (Godefroy-Colburn and Grunberg-Manago, 1972), RNA polymerase (Chamberlin, 1976) and possibly DNA polymerase (Lehman, 1981).

We return to the simplest kinetic scheme posed, i.e., that leading to the Poisson distribution of eq'n (4). We then ask how the inclusion of the possibility of reassociation of a given enzyme molecule with the same chain to which it has just catalyzed the addition of one monomeric

unit will affect the kinetics of subsequent reaction. An overly lengthy kinetic analysis (Peller, 1984) shows that a Poisson distribution will also arise but with ν replaced by $\nu(1-\beta)$. Here β is the fractional ratio of the unimolecular rate constant for reaction caused by reassociation to the sum of that constant and the other unimolecular rate constants involved with the formation and breakdown of a given enzyme n-mer complex.

As $\beta \to 1$, the constant in the exponent of the Gaussian limit for this Poisson distribution in eq'n $\nu(1-\beta)$ becomes smaller and the size distribution correspondingly widens. This action of a probability after-effect (Chandrasekhar, 1943) is quite readily comprehensible arising as it does from the possibility of the enzyme disassociating from the macromolecule after catalyzing a variable number of additions of monomer instead of after each monomeric accretion. To meld this process with the other complications discussed previously seems impossible without extensive numerical analysis and machine computation.

There are presently available from the International Mathematics and Statistics Library subroutines suitable for mini computers produced by the Data General Corporation and the Digital Equipment Corporation capable of dealing with large systems of non-linear differential equations. It is hoped to exploit these either through fairly heavily used local computers or by a modem to an Air Force facility.

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INTERNATIONAL SYMPOSIUM

Toronto, November 1985, HPLC of Polypeptides and Nucleic Acids. Participants

Hakam, A. and Kun, E.

Four seminars:	Cancer Institute Lausanne
(E. Kun)	University of Zurich
	University of Innsbruck
	University of Freiburg